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(71) Applicant *(for all designated States except US)*: **UNIVERSITY OF FLORIDA RESEARCH FOUNDATION, INC.** [US/US]; 223 Grinter Hall, Gainesville, FL 32611-5500 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **MUZYCZKA, Nicholas** [US/US]; 9837 S.W. 67th Drive, Gainesville, FL 32608 (US). **BURGER, Corinna** [ES/US]; 1724 nw 12th Street, Gainesville, FL 32608 (US). **HAUSWIRTH, William, W.** [US/US]; 12801 S.W. 39th Street, Gainesville, FL 32608 (US). **MANDEL, Ronald, J.** [US/US]; 6525 NW 81st Blvd., Gainesville, FL 32653 (US). **ZOLOTUKHIN, Sergei** [US/US]; 1122 S.W. 96th Street, Gainesville, FL 32607 (US).

(74) Agent: **MOORE, Mark, D.**; Williams, Morgan & Amerson, P.C., 10333 Richmond, Suite 1100, Houston, TX 77042 (US).

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(54) Title: IMPROVED RAAV EXPRESSION SYSTEMS AND METHODS FOR ENHANCING TRANSDUCTION OF MAMMALIAN NEURAL CELLS

(57) Abstract: Disclosed are serotype-specific recombinant adeno-associated viral (rAAV) vectors, as well as viral particles and compositions comprising them, useful in the expression of neurotherapeutic agents (including neurotherapeutic peptides and polypeptides) in selected mammalian neural cells, as well as tissues and organ systems comprising them. In particular embodiments, rAAV serotype 1 and serotype 5 vectors are disclosed useful for the delivery of therapeutic agents to neural cells of affected mammals.

DESCRIPTION

IMPROVED RAAV EXPRESSION SYSTEMS AND METHODS FOR ENHANCING TRANSDUCTION OF MAMMALIAN NEURAL CELLS

1. BACKGROUND OF THE INVENTION

The present application claims priority to United States Provisional Patent Application Serial No. 60/377,313 filed May 1, 2002, the entire contents of which is specifically incorporated herein by reference in its entirety. The United States government has certain rights in the present invention pursuant to grant number NS36302 from the National Institutes of Health.

1.1 FIELD OF THE INVENTION

The present invention relates generally to the fields of molecular biology and virology, and in particular, to the development of gene delivery vehicles for transduction of mammalian cells. The invention concerns the use of particular rAAV serotypes in a variety of investigative, diagnostic and therapeutic regimens, including for example, the treatment of neurological disorders and diseases of the nervous system. Methods and compositions are also provided for preparing rAAV serotype-specific vector constructs that are useful in promoting the expression of one or more therapeutic agents to selected mammalian cells.

1.2 DEFICIENCIES IN THE PRIOR ART

Currently, there are limited gene-therapy approaches to treating neurological disorders and diseases of the nervous system in an affected animal using adeno-associated viral delivery vectors. AAV2 serotypes, for example, are deficient for transducing some types of neuronal cells. Cells of the hippocampal regions CA1, CA2, and CA3, which are involved in learning and memory, Alzheimer's disease, and epilepsy, for example, are not efficiently transduced with AAV2 serotype vectors. Many current methods, also introduce undesirable side-effects, and do not overcome the problems associated with traditional modalities and treatment regimens for such conditions. Thus, the need exists for an effective treatment that circumvents the adverse effects and provides more desirable results, with longer acting effects, and improved patient compliance. In addition, methods for viral delivery of polynucleotides to neural cells and tissues that express one or more genes encoding a neurotherapeutic polypeptide are desirable that are useful in the amelioration of such conditions, and in particular, administration of

serotype-specific rAAV vectors comprising polynucleotide constructs to neural cells and tissues to treat such conditions are particularly desirable.

2. SUMMARY OF THE INVENTION

The present invention overcomes these and other limitations inherent in the prior art by providing new serotype-specific rAAV-based genetic constructs that encode one or more mammalian neurotherapeutic peptides, proteins, polypeptides, antibodies, antigen binding fragments, enzymes, catalytic RNA ribozyme molecules, or antisense molecules for the prevention, treatment, and/or amelioration of symptoms of one or more neurological disorders and/or diseases of the brain, nervous system, or neural tissues that result either from a deficiency in, or the absence of a biologically-active form of one or more of such peptides, polypeptides, or proteins, or in the case of therapy with antisense and ribozyme molecules, where the disease or dysfunction arises from the expression or overexpression of one or more native or mutated proteins, peptides or polypeptides.

In the case of a peptide, protein, or polypeptide deficiency, the present rAAV expression systems typically will comprise an expression unit that encodes the deficient or defective peptide, protein, or polypeptide, thereby providing therapeutically-effective amounts to the cells in need thereof. In the case of an overexpression of one or more native peptides, proteins, or polypeptides, or the production of an aberrant or mutant peptide, protein, or polypeptide, the vectors of the invention most preferably will encode a catalytic RNA molecule or antisense molecule designed, such that, when the vector is expressed in such cells, the antisense molecule binds to, or the ribozyme molecules catalytically cleaves the mRNA that is producing the aberrant or defective protein, thereby reducing the level of the native peptide, protein, or polypeptide, to a level wherein the disease or dysfunction is now ameliorated, treated, or prevented.

The invention provides serotype-specific AAV-based genetic constructs encoding one or more expressed mammalian neurotherapeutic agents and compositions comprising them for use in the treatment of a variety of conditions and mammalian diseases and disorders involving the cells, tissues, and/or organs of the mammalian nervous system.

The present invention overcomes these and other limitations inherent in the prior art by providing new rAAV-based genetic expression constructs specifically suited for transforming mammalian cells, and in particular neural cells. The rAAV expression systems and vectors of the invention preferably encode at least a first neurotherapeutic agent, and in particular, at least a first neurotherapeutic antisense molecule, ribozyme, protein or polypeptide that is useful in the

amelioration of symptoms, treatment, and/or prevention of certain types of mammalian diseases and dysfunctions, including, for example, neural and neuromuscular dysfunction and other diseases of neural cells and tissues and organ systems comprising such neural cells.

When expressed in mammalian neural cells, the neurotherapeutic agents of the present invention may act by providing a beneficial result or outcome, for example, to promote the restoration of function and/or to promote or enhance the longevity of the neuronal cells. Such cells may be cells that are apparently deficient in a normal phenotype (such as for example, the loss of function, or susceptibility to premature cell death) or alternatively, the neurotherapeutic agent may enhance function or prolong longevity in neuronal cells with an apparently normal phenotype, function or longevity profile. The therapeutic benefit may be achieved by, or results from, expression of an agent that positively stimulates or promotes function or longevity. Alternatively, the therapeutic benefit may be achieved by, reducing or inhibiting the expression or biological activity of a component or pathway that is detrimental to the normal function or lifespan of such cells. In certain cases, for example, in the expression of an antisense or a ribozyme molecule, the therapeutic agent may achieve the desired result by inhibiting the transcription or translation of one or more genes in the cells that when expressed, result in an altered phenotype, or in the accumulation of one or more intracellular components that impair or prevent normal cellular function and/or cell lifespan.

In one embodiment, the invention provides an adeno-associated viral vector comprising at least a first polynucleotide that comprises a promoter operably positioned upstream of an isolated nucleic acid segment encoding a biologically-active neurotherapeutic mammalian peptide, protein, polypeptide, antisense molecule or ribozyme wherein the promoter expresses the nucleic acid segment in a mammalian cell that comprises the vector to produce the encoded mammalian peptide, protein, polypeptide, antisense or ribozyme. In the case of neurotherapeutic peptides, proteins, or polypeptides, the construct preferably encodes a biologically active protein peptide or polypeptide selected from the group consisting of a neurotrophic factor, a cytokine, a cytotoxin, a tumor suppressor, an anti-apoptotic factor, a growth factor, a cytokine receptor, a growth factor receptor, an interferon, a semaphorin, a semaphoring receptor, a plexin, a plexin receptor, a neuropilin, a neuropilin receptor, a netrin, a netrin receptor, a serotonin transport protein, a hormone receptor, a decarboxylase, a protein kinase, a protein kinase inhibitor, a glycoprotein, a hormone, a proteolytic protein, a neurogenic factor, a growth factor, a neurotrophin, an apoptosis inhibitor, an adrenergic agonist, an erythropoietic agent, an N-methyl-D-aspartate antagonist, a nerve growth factor, a neuroactive

peptide receptor, a neurotrophin receptor, or other neurologically-beneficial expressed biologically-active therapeutic. Exemplary neurotherapeutic polypeptides include, but are not limited to, those selected from the group consisting of BDNF, CNTF, CSF, EGF, enolase, FGF, glutamic acid decarboxylase, G-SCF, GM-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, interleukin receptor, IFN, IFG-1, M-CSF, MDK, neuropilin 1, neuropilin 2, neurotrophin 1, neurotrophin 2, neurotrophin 3, neurotrophin 4, neurotrophin 5, neurotrophic tyrosine kinase, NGF receptor, NGF, NGF receptor associated protein, NGFB, PDGF, PDGF receptor, PEDF, PEDF receptor, TGF, TGF receptor, TGF-B2, TNF, TNF receptor, VEGF, and VEGF receptor to name only a few.

Examples of neurotrophic polypeptide sequences, and the nucleic acid sequences that encode them include, but are not limited to, anti-apoptotic polypeptides, cytokines, growth factors, neurotrophins, neurotrophic factors, neurotrophin receptors, growth factor receptors, cytokine receptors, including but not limited to human CNTF (GenBank Accession number NM_001842) BDNF (GenBank Accession number XM_006027) GDNF (GenBank Accession number NM_001485), GDNF-beta (GenBank Accession number U93703), FGFR (GenBank Accession number NM_006533), NGF (GenBank Accession number AF411526), NGF receptor-associated protein (GenBank Accession number XM_055898), neurotrophin 6 gamma (GenBank Accession number S41541), neurotrophin 6 beta (GenBank Accession number S41540), neurotrophin 6 alpha (GenBank Accession number S41522), nerve growth factor receptor (GenBank Accession number NM_014380), neurotrophin 5 (GenBank Accession number NM_006179), neurotrophin 4 (GenBank Accession number M86528), neurotrophin 3 (GenBank Accession number NM_002527), Human neurotrophic tyrosine kinase type 2 receptor (GenBank Accession number NM_006180); Human nerve growth factor receptor associated protein 1 (GenBank Accession number NM_014380); Human nerve growth factor receptor (GenBank Accession number NM_002507); Human neurotrophin 5 (GenBank Accession number NM_006179); Human neurotrophin 3 (GenBank Accession number NM_002527); Human neurotrophin 6 (GenBank Accession number S41540); Human neurotrophin receptor tyrosine kinsase type 2 (GenBank Accession number AF410901); Human neuropilin 2 (GenBank Accession number NM_018534); Human neuropilin 1 (GenBank Accession number NM_003873); Human VEGF (GenBank Accession number NM_003376); Human BDNF (GenBank Accession number AF411339); Human VGF (GenBank Accession number NM_003378); Human nerve growth factor (GenBank Accession numbers NM_002507 and NM_002506); Human NGFB (GenBank Accession number AF411526); Human EGF (GenBank Accession number NM_001963);

Human MDK (GenBank Accession number NM_002391); Human FGF (GenBank Accession number NM_002006), and neurotrophin 1 (GenBank Accession number AF176911), each of which is specifically incorporated herein by reference in its entirety.

The adeno-associated viral vectors of the invention typically comprise at least a first promoter, such as a heterologous, tissue-specific, constitutive or inducible promoter, that can express in mammalian, and particularly, human cells and tissues, and most particularly in neural cell, neural tissues, and organs such as the brain and tissues of the central and peripheral nervous systems. Exemplary such promoters, for example, include brain-specific, neural cell-expressible, or nerve tissue-specific promoters. Exemplary heterologous promoters include, but are not limited to, those selected from the group consisting of a CMV promoter, a β-actin promoter, a hybrid CMV promoter, a hybrid β-actin promoter, an EF1 promoter, a U1a promoter, a U1b promoter, a Tet-inducible promoter and a VP16-LexA promoter.

The genetic constructs of the invention may also further optionally comprise one or more enhancer sequences operably linked to the nucleic acid segment to enhance expression of the encoded therapeutic agent in certain cell types. Exemplary enhancer sequences, include, but are not limited to, a CMV enhancer, a synthetic enhancer, a brain-specific enhancer, a neural cell-specific enhancer, or a nerve cell-specific enhancer.

The rAAV vectors may also further optionally comprise one or more post-transcriptional regulatory sequences, such as the woodchuck hepatitis virus post-transcription regulatory element.

The invention also provides recombinant adeno-associated virus virions and pluralities of rAAV viral particles that comprise at least a first neurotherapeutic AAV construct as disclosed herein. While the rAAV particles of the invention may be of any of the known serotypes, such as for example, AAV serotype 1, AAV serotype 2, AAV serotype 3, AAV serotype 4, AAV serotype 5, and AAV serotype 6, virions of the 1st and 5th serotypes are particularly contemplated to be useful in the practice of the invention. Likewise, those rAAV particles that have been modified on their surface by expression of one or more targeting sequences, or by the deletion of one or more targeting sequences, such that they have an altered serotype, or an altered affinity for particular cell types (when compared to the native, unmodified virion) are also contemplated to be useful in the present invention. For example, while native rAAV2 virions do not typically transfect neural cells with significant affinity, a modified rAAV2 vector that has an altered capsid protein profile, for example, may have significantly enhanced ability to transfect neural cells. Such modified AAV

vectors and virions also form important aspects of the invention. In fact, by genetic modification through the hand of man, virtually any known or identified AAV virion may be recombinantly engineered such that the virus has altered or improved affinity for particular cell types when compared to its native unmodified form. Likewise, it may be desirable to modify the AAV virions of the invention to improve penetration of the blood-brain barrier, or such like.

A further aspect of the invention concerns mammalian cells, and isolated host expression cells that comprise at least one of the rAAV vectors, virions, or viral particles disclosed herein. Although all mammalian cells are contemplated to be useful in the practice of the present invention, in certain embodiments, mammalian cells include, endothelial cells, neural cells, glial cells, vascular cells, dendritic cells, and such like will be particularly useful. Preferably such mammalian cells are human cells, although when veterinary applications of the disclosed technology are desired, the host cells may be of any mammalian species, including for example, primates, rodents, ungulates, commercially-useful livestock animals, pets, domesticated animals, and such like.

As described hereinbelow, the invention also provides compositions and kits that comprise one or more of the disclosed vectors, virions, viral particles, or host cells of the invention. Typically such compositions will further comprise at least a first pharmaceutical excipient, buffer, or diluent, and may be formulated for administration to a human, or an animal under veterinary care. Such compositions may further optionally comprise one or more additional therapeutic compounds, compositions, or medicaments, and may be formulated for use in the prophylaxis or therapy of a variety of diseases, disorders, or dysfunctions, such as, for example, for use in cancer, autoimmune disease, neuromuscular disease, neurosensory impairment, cognitive or memory impairment, mental dysfunction, or other neural disease or dysfunction for which neurotherapeutic treatment modalities are contemplated.

The compositions as disclosed herein may further comprise at least a first liposome, lipid, lipid complex, microsphere, microparticle, nanosphere, or nanoparticle, as may be desirable to facilitate or improve delivery of the particular neurotherapeutic agent to one or more cell types, tissues, or organs in the animal to be treated.

In addition to the vectors, compositions, host cells, and kits described above, the invention also pertains to the use of such compositions in the treatment and/or prophylaxis of a number of diseases and dysfunctions of neural or nervous system origin. In a general sense, the methods of the invention concern means for preventing, treating or ameliorating

the symptoms of a disease, dysfunction, or deficiency in a mammal. The methods generally involve providing to or administering to the mammal a composition that comprises the virions or the viral particles as disclosed herein in an amount and for a time sufficient to treat or ameliorate the symptoms of the disease, dysfunction, or deficiency in the mammal. In illustrative embodiments, the mammal has, is diagnosed with, or is at risk for developing, a neural impairment, a neurosensory disorder, a neuromuscular disease, a cancer, tumor, or metastatic growth or lesion of the brain or nervous system, memory loss, age-related memory loss, neurocognitive disease, a socioaffective disorder, autism, ALS, cerebral palsy, ischemia and cerebrovascular injuries, Alzheimer's, Parkinson's, Huntington's, Tay-Sach's, Niemann-Pick's Guillain-Barre syndrome, seizures, coma, dementia, schizophrenia, brain insult or injury, comprehension or learning disabilities, sensory motor impairment, to name only a few.

Although all mammals may find benefit of the present invention, in preferred embodiments, the animal is a human being that has, has been diagnosed with, or is at risk for developing one or more such disorders.

In the methods of the invention, the virions or plurality of viral particles, or one or more compositions comprising them are provided to, or administered to, the mammal by a suitable delivery means. Exemplary means for delivering rAAV particles to a mammal, include, for example, by intramuscular, intravenous, subcutaneous, intrathecal, intraperitoneal, or intracerebroventricular administration, or by direct injection into one or more tissues or organs, such as for example, by injection into the tissues or cells of the brain or spinal cord, into a bone or joint, or, into the muscles or subcutaneous spaces, or vascular tissues of the mammal.

The invention also provides a method for preventing, treating, or ameliorating the symptoms of a neural, neuromuscular, or central nervous system disease or dysfunction in a mammal suspected of having, or at risk for developing such a condition. The method generally involves providing to such a mammal one or more of the therapeutic rAAV compositions disclosed herein, in an amount and for a time sufficient to treat or ameliorate the symptoms of the disease or dysfunction in the mammal. Preferred animals include those under veterinary care, as well as human beings under the care of a physician, and particularly those with a familial history of such disease or dysfunction, or those at risk for developing it.

The vector constructs of the present invention may also further optionally comprise one or more native, synthetic, or hybrid enhancer elements, for example, a CMV enhancer, a synthetic enhancer, or a tissue- or cell-specific enhancer, such as for example, a neural cell,

dendritic cell, or a nerve cell-specific promoter, to improve the expression of the therapeutic construct in a particular cell type.

The vector constructs of the present invention may also further optionally comprise one or more native, synthetic, or hybrid post-transcriptional regulatory elements that may function to help stabilize the RNA and increase overall expression of the therapeutic polypeptide. An exemplary such element is the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) (for examples, see Paterna *et al.*, 2000 and Loeb *et al.*, 1999).

The vectors may also further optionally comprise one or more intron sequences to facilitate improved expression of the therapeutic genes placed under the control of the promoter and/or promoter/enhancer regulatory regions. The vector systems of the invention may also include "helper" plasmids that provide necessary auxiliary functions required for AAV infection and/or propagation, such as for example, viral Rep and Cap sequences, and the terminal repeat elements from AAV. Such components may be on a single plasmid or expression system, or alternatively, such functions may reside on separate, distinct plasmids, providing for a multi-component AAV expression system. The use of "helper" plasmids and multi-component AAV expression systems have been routinely employed by the inventors, and are widely-understood by skilled artisans in the field.

The vectors and compositions that comprise one or more of the novel vectors disclosed herein may be provided to a mammal through suitable administration means, such as, by intramuscular, intravenous, intrathecal, intracerebroventricular, or direct injection to one or more sites in the brain, spinal cord, neural cells, tissues, or the central nervous system in selected mammals. Typically, such compositions will be formulated with pharmaceutically-acceptable excipients as described hereinbelow, and may comprise one or more liposomes, lipids, lipid complexes, microcapsules, microspheres, nanospheres, or nanoparticle formulations to facilitate administration to the selected organs, tissues, and cells for which therapy is desired, such as, for example, in selected neural or neuromuscular tissues and the like.

Another important aspect of the present invention concerns methods of use of the disclosed vectors, virions, compositions, and host cells described herein in the preparation of medicaments for treating or ameliorating the symptoms of various neurological conditions resulting from a defect of one or more neurotherapeutic polypeptide in a mammal. Such methods generally involve administration to a mammal (and in particular, a human being in need thereof), one or more of the disclosed vectors, virions, host cells, or compositions, in an amount and for a time sufficient to treat or ameliorate the symptoms of such a deficiency in

the affected mammal. The methods may also encompass prophylactic treatment of animals suspected of having such conditions, or administration of such compositions to those animals at risk for developing such conditions either following diagnosis, or prior to the onset of symptoms. The invention also provides methods and compositions for the expression of therapeutic polypeptides not normally expressed in neural tissues through the use of neural-specific, or nerve cell-active promoters (*e.g.*, neural-specific enolase promoter [Sakimura *et al.*, 1993]), enhancers, and other neural regulatory elements that may be operably positioned to facilitate expression of one or more therapeutic polypeptides in such cells.

In certain embodiments, the vectors of the present invention may be modified to include one or more targeting sequences that, when expressed on the surface of the rAAV virions, causes the virions to have an increased affinity for binding to, or for transducing, one or more cells that comprise a receptor on their surface for which the targeting sequence on the virion has an affinity for binding to. Such sequences are particularly desirable when the particular rAAV serotype employed does not target or bind to such cells naturally with sufficient affinity to affect therapy, prophylaxis, or treatment. For example, by providing one or more small peptide epitope ligands on the surface of the rAAV virions, one may achieve a higher binding affinity for, or a higher transduction efficiency for a particular neural or nervous system cell, than the native unmodified rAAV virion has for such cells. Modification of such virions may be accomplished as described herein by altering one or more of the rAAV capsid proteins to contain a targeting ligand that provides such rAAV virions with an increased ability to selectively bind to a given cell type.

The invention also provides serotype-specific rAAV vector compositions that have been shown to be surprisingly effective in successfully transfecting neural cells and nervous system tissues. This invention extends the current usefulness of rAAV-based gene therapy methods to provide for better vectors useful in the therapy, prophylaxis, or treatment of one or more neurological and neuromuscular diseases, dysfunctions, and disorders.

In one embodiment, the invention provides a serotype-specific adeno-associated viral vector that comprising at least a first polynucleotide that encodes a neurotherapeutic peptide or polypeptide operably linked to a nucleic acid segment that comprises at least a first promoter capable of expressing the nucleic acid segment in a nerve cell, a brain cell, or a cell of the ventral or peripheral nervous system that comprises the particular vector. In preferred embodiments, the nucleic acid segment encodes a mammalian, and in particular, a human neurotherapeutic agent. As used herein, a neurotherapeutic agent is any expressible biological molecule (such as a peptide, protein, polypeptide, antisense molecule, or ribozyme), that

Alternatively, the neurotherapeutic constructs of the invention may encode polypeptides of murine, porcine, bovine, ovine, feline, canine, equine, epine, caprine, or lupine origin. In illustrative embodiments, the vector may also optionally comprise one or more neural cell-targeting sequences that enhance the targeting of the specific rAAV construct to a neural cell to provide therapeutic levels of the selected protein to the transfected neural cells.

The inventors have made the surprising discovery that particular rAAV serotypes, namely rAAV serotype 1 (rAAV1) and rAAV serotype 5 (rAAV5), have a surprisingly enhanced affinity for transfection of neural cells when compared to other rAAV serotypes such as rAAV serotype 2 (rAAV2), rAAV serotype 3 (rAAV3), rAAV serotype 4 (rAAV4), or rAAV serotype 6 (rAAV6). In fact, for some neural cell types, AAV1 and AAV5 vectors have been shown to be 1000-fold more effective in transfecting neural cells than the more commonly-used AAV2 vectors.

The vector constructs of the invention preferably comprise at least a first constitutive or inducible promoter that is capable of expressing the neurotherapeutic polypeptide-encoding DNA sequence of interest in mammalian neural cells infected with the viral vector. In exemplary embodiments, such promoters selected from the group consisting of a CMV promoter, a β-actin promoter, a hybrid CMV promoter, a hybrid β-actin promoter, an EF1 promoter, a U1a promoter, a U1b promoter, a Tet-inducible promoter and a VP16-LexA promoter are particularly useful in the practice of the invention.

Likewise, neural tissue-specific or neural-cell-specific promoters also form part of the invention in the expression of selected neurotherapeutic polypeptide-encoding DNA segments in a suitably transformed mammalian neural cell. Such neural tissue specific promoters may be selected from the group consisting of, but not limited to, neurotrophic factor-derived promoters, nerve growth factor-derived promoters, and the like.

In illustrative embodiments, at least a first polynucleotide encoding a neurotherapeutic protein, polypeptide or peptide is operably positioned under the control of the selected promoter and/or neural enhancer element(s) and used to produce neurotherapeutically effective levels of the encoded polypeptide when suitable host neural cells and/or tissues are transformed with the serotype-specific rAAV genetic constructs disclosed herein.

The vector constructs of the present invention may also further optionally comprise one or more native, synthetic, or hybrid enhancer elements, for example, a CMV enhancer, a synthetic enhancer, or a tissue- or cell-specific enhancer, such as for example, a nerve cell, or an neural-cell-specific promoter and/or enhancer, such as a human neurotrophic factor promoter or enhancer.

The vector constructs of the present invention may also further optionally comprise one or more native, synthetic, or hybrid post-transcriptional regulatory elements that may function to help stabilize the RNA and increase overall expression of the therapeutic polypeptide. An exemplary such element is the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) (see Paterna *et al.*, 2000 and Loeb *et al.*, 1999).

The vectors may also further optionally comprise one or more intron sequences to facilitate improved expression of the neurotherapeutic gene(s) placed under the control of the promoter and/or promoter/enhancer regulatory regions to increase or enhance expression of the selected gene of interest in particular neural cells or tissues of the brain, CNS or PNS.

The vectors may also further optionally comprise at least a second polynucleotide that encodes one or more targeting sequences to facilitate improved targeting of the rAAV constructs and rAAV virions to the particular neural cells or tissues of the brain, CNS or PNS.

As described herein, the use of AAV1- or AAV5-vectored ribozymes for treatment of neural diseases also forms an important aspect of the present invention. Such catalytic RNA molecules may be of the hairpin or the hammerhead variety. A further aspect of the invention is a vector, virus, or host cell that comprises a polynucleotide encoding one or more such preferred ribozymes. Such vectors, virus and host cells will preferably comprise at least a first such polynucleotide that is operably linked to at least a first promoter element that directs expression of the polynucleotide in a mammalian neural cell to produce the desired ribozyme.

Other aspects of the invention concern recombinant adeno-associated virus virion particles, compositions, and host cells that comprise one or more of the serotype-specific rAAV vectors disclosed herein, such as for example pharmaceutical formulations of the vectors intended for administration to a mammal through suitable means, such as, by intramuscular, intravenous, or direct injection to one or more cells, tissues, organs, or organ systems of a selected mammal, and in the case of delivery to the brain and its tissues, stereotactic injection directly to one or more tissues of the brain itself is particularly preferred. Typically, such compositions will be formulated with pharmaceutically-acceptable excipients as described hereinbelow, and may comprise one or more liposomes, lipids, lipid complexes, microspheres or nanoparticle formulations to facilitate administration to the selected organs, tissues, and cells for which therapy is desired, such as for example, in the formulation of compositions suitable for crossing the blood:brain barrier, *etc.*.

Therapeutic and diagnostic kits also form important aspects of the present invention. Such kits typically comprise one or more of the disclosed AAV vector constructs, virion particles, or therapeutic compositions described herein, and instructions for using the kit. Such

kits may comprise a plurality of vectors, a plurality of virions, either alone, or in combination with one or more additional therapeutic agents as may be desired for effective therapies.

Another important aspect of the present invention concerns methods of use of the disclosed vectors, virions, compositions, and host cells described herein in the preparation of medicaments for treating or ameliorating the symptoms of such a disease or dysfunction, or other conditions resulting from a deficiency in one or more peptides or polypeptides in a mammal. Such methods generally involve administration to a human in need thereof, one or more of the disclosed rAAV vectors, virions, host cells, or pharmaceutical compositions, in an amount and for a time sufficient to treat or ameliorate the symptoms of such a deficiency in the affected animal. The methods may also encompass prophylactic treatment of animals suspected of having such conditions, or administration of such compositions to those animals at risk for developing such conditions either following diagnosis, or prior to the onset of symptoms. Such symptoms may include, but are not limited to, neural dysfunction, neurological disorder, neuromuscular disease, neurodegeneration, and a variety of diseases and disorders of the brain, central nervous system (CNS) (including the brain and the spinal cord), or the peripheral nervous system (PNS) (including the cranial and peripheral nerves and associated ganglia), such as for example, damage, trauma, dysfunction, or other defect in the neurons, dendrites, axons, striatum, or other cellular components of the mammalian nervous system, including components of the brain such as the cerebral hemispheres, the cerebellum, and the brain stem, the latter of which includes the diencephalon; mesencephalon, or midbrain; pons; and the medulla oblongata.

The methods may also encompass treatment of animals suspected of having such conditions as epilepsy, Alzheimer's disease, Parkinson's disease, or other dysfunctions affecting short-term, intermediate-term, or long-term memory and the like. The inventors have made the surprising discovery that AAV1 and AAV5 serotype vectors are able to transduce neural cells to a degree of approximately 1000-fold more efficient than other AAV serotypes such as AAV2. This is particularly useful in the transduction of the hippocampal region of the brain that contains the principal neurons believed to be involved in the formation of memory. Particularly advantageous is the use of the present compositions and methods to transfect pyramindal neurons of the cingulate and dentate gyrus regions of the hippocampus, including the stratum radiatum, and particularly the regions known as CA1, CA2, and CA3.

The present invention also concerns methods for high-level delivery of rAAV serotype-specific vectors to neural tissues useful in delivery of polypeptides useful in the therapy of tumors and cancers of the brain, including, for example, gliomas, astrocytomas, and the like.

3. BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to the following description taken in conjunction with the accompanying drawings, in which like reference numerals identify like elements, and in which:

FIG. 1A-FIG. 1H. Comparison of striatal transduction efficiency of rAAV5-CBA-GFP (**FIG. 1A, FIG. 1B, FIG. 1C, FIG. 1D, and FIG. 1H**) versus rAAV2-CBA-GFP (**FIG. 1E, FIG. 1F, FIG. 1G, and FIG. 1I**) with photomicrographs of native GFP fluorescence. Both vectors were injected into striatum as 2×10^{10} particles in 2 µl. **FIG. 1A-FIG. 1D** represent the extent of striatal rAAV5-CBA-GFP transduction with **FIG. 1A** being the most anterior section and **FIG. 1D** being the most posterior. **FIG. 1E-FIG. 1G** demonstrates the extent of striatal transduction obtained from rAAV2-CBA-GFP (**FIG. 1E-FIG. 1G**, anterior-posterior). **FIG. 1G** was digitally enhanced to reveal the small transduction area as indicated by the arrowheads. FIG. 1H and FIG. 1I are higher power photomicrographs from rAAV5-CBA-GFP and rAAV2-CBA-GFP respectively. Bars in **FIG. 1B and FIG. 1F** = 500 µm applies to **FIG. 1A-FIG. 1G**, Bars, in **FIG. 1H** and **FIG. 1I** = 100 µm cc = corpus callosum, LV = lateral ventricle, ac = anterior commisure.

FIG. 2A and FIG. 2B. Hippocampal comparison AAV2 *vs.* AAV5. Higher quality comparison between the hippocampal transduction efficiency of rAAV2 *vs.* rAAV5 both expressing GFP under the control of the CBA promoter. A single introhippocampal injection of 2 µl of rAAV-GFP in a rat (FIG. 2B) lead to GFP positive neurons in all the CA regions and the dentate gyrus, while an identical rAAV2-GFP injection (FIG. 2A, white arrow) was much less effective in mediating GFP expression in hippocampus. While the UF vector core is continuing to develop methods to correctly purify rAAV5, this vector serotype will ultimately be available for use on this project.

FIG. 3A, FIG. 3B, FIG. 3C, and FIG. 3D. AAV1/str refers to a single 2 µl injection of rAAV1-GFP in the striatum (FIG. 3A), AAV2/str refers to a single 2 µl injection of rAAV2-GFP in the striatum (FIG. 3B), AAV1/hpc refers to a single 2 µl injection of rAAV1-GFP in the hippocampus (FIG. 3C) and AAV2/hpc refers to a single 2 µl injection of rAAV2-GFP in the hippocampus (FIG. 3D). The magnifications are the same in all four panels displaying about a 2.5 × 3 mm cross-section.

FIG. 4A, FIG. 4B, FIG. 4C, and FIG. 4D. AAV1/GP refers to a single 2 μl injection of rAAV1-GFP in the globus pallidus (FIG. 4A), AAV2/GP refers to a single 2 μl injection of rAAV2-GFP in the globus pallidus (FIG. 4B), AAV1/sn refers to a single 2 μl injection of rAAV1-GFP in the substantia nigra (FIG. 4C) and AAV2/sn refers to a single 2 μl injection of rAAV2-GFP in the substantia nigra (FIG. 4D). The magnifications are the same in all four panels displaying about a 2.5 × 3 mm cross-section.

4. DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Illustrative embodiments of the invention are described below. In the interest of clarity, not all features of an actual implementation are described in this specification. It will of course be appreciated that in the development of any such actual embodiment, numerous implementation-specific decisions must be made to achieve the developers' specific goals, such as compliance with system-related and business-related constraints, which will vary from one implementation to another. Moreover, it will be appreciated that such a development effort might be complex and time-consuming, but would nevertheless be a routine undertaking for those of ordinary skill in the art having the benefit of this disclosure.

4.1 PHARMACEUTICAL COMPOSITIONS

The genetic constructs of the present invention may be prepared in a variety of compositions, and may also be formulated in appropriate pharmaceutical vehicles for administration to human or animal subjects. The AAV molecules of the present invention and compositions comprising them provide new and useful therapeutics for the treatment, control, and amelioration of symptoms of a variety of disorders. Moreover, pharmaceutical compositions comprising one or more of the nucleic acid compounds disclosed herein, provide significant advantages over existing conventional therapies – namely, (1) their reduced side effects, (2) their increased efficacy for prolonged periods of time, (3) their ability to increase patient compliance due to their ability to provide therapeutic effects following as little as a single administration of the selected therapeutic AAV composition to affected individuals. Exemplary pharmaceutical compositions and methods for their administration are discussed in significant detail hereinbelow.

The invention also provides compositions comprising one or more of the disclosed vectors, expression systems, virions, viral particles; or mammalian cells. As described hereinbelow, such compositions may further comprise a pharmaceutical excipient, buffer,

or diluent, and may be formulated for administration to an animal, and particularly a human being. Such compositions may further optionally comprise a liposome, a lipid, a lipid complex, a microsphere, a microparticle, a nanosphere, or a nanoparticle, or may be otherwise formulated for administration to the cells, tissues, organs, or body of a mammal in need thereof. Such compositions may be formulated for use in therapy, such as for example, in the amelioration, prevention, or treatment of conditions such as peptide deficiency, polypeptide deficiency, tumor, cancer or other malignant growth, neurological dysfunction, autoimmune disease, neural diseases, including Alzheimer's, and Parkinson's, memory loss, motor impairment, and the like, as well as liver disease or dysfunction, and musculoskeletal diseases including, for example, arthritis, ALS, MLS, MD, and such like, to name only a few.

In certain embodiments, the present invention concerns formulation of one or more of the rAAV compositions disclosed herein in pharmaceutically acceptable solutions for administration to a cell or an animal, either alone or in combination with one or more other modalities of therapy, and in particular, for therapy of mammalian neural cells, neurological tissues, and diseases affecting the brain, CNS, or peripheral nervous system.

It will also be understood that, if desired, nucleic acid segments, RNA, DNA or PNA compositions that express one or more of neurotherapeutic gene products may be administered in combination with other agents as well, such as, *e.g.*, proteins or polypeptides or various pharmaceutically-active agents, including one or more systemic or topical administrations of therapeutic polypeptides, biologically active fragments, or variants thereof. In fact, there is virtually no limit to other components that may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the target cells or host tissues. The rAAV compositions may thus be delivered along with various other agents as required in the particular instance. Such compositions may be purified from host cells or other biological sources, or alternatively may be chemically synthesized as described herein. Likewise, such compositions may further comprise substituted or derivatized RNA, DNA, or PNA compositions.

Formulation of pharmaceutically-acceptable excipients and carrier solutions is well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including *e.g.*, oral, parenteral, intravenous, intranasal, and intramuscular administration and formulation.

Typically, these formulations may contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 70% or 80% or more of the weight or volume of the total formulation. Naturally, the amount of active compound(s) in each therapeutically-useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

In certain circumstances it will be desirable to deliver the AAV vector-based therapeutic constructs in suitably formulated pharmaceutical compositions disclosed herein either subcutaneously, intraocularly, intravitreally, parenterally, intravenously, intracerebroventricularly, intramuscularly, intrathecally, orally, intraperitoneally, by oral or nasal inhalation, or by direct injection to one or more neural cells, nervous tissues, or even by direct injection or administration to the brain, CNS or to the peripheral nervous system. The methods of administration may also include those modalities as described in U. S. Patent 5,543,158; U. S. Patent 5,641,515 and U. S. Patent 5,399,363 (each specifically incorporated herein by reference in its entirety). Solutions of the active compounds as freebase or pharmacologically acceptable salts may be prepared in sterile water and may also suitably mixed with one or more surfactants, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms of the AAV-based viral compositions suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U. S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

The prevention of the action of microorganisms can be brought about by various antibacterial ad antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

For administration of an injectable aqueous solution, for example, the solution may be suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, and the general safety and purity standards as required by FDA Office of Biologics standards.

Sterile injectable solutions are prepared by incorporating the active AAV vector-delivered therapeutic polypeptide-encoding DNA fragments in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The AAV vector compositions disclosed herein may also be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric

hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug-release capsules, and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human, and in particular, when administered to the human eye. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

The amount of AAV compositions and time of administration of such compositions will be within the purview of the skilled artisan having benefit of the present teachings. It is likely, however, that the administration of therapeutically-effective amounts of the disclosed compositions may be achieved by a single administration, such as for example, a single injection of sufficient numbers of infectious particles to provide therapeutic benefit to the patient undergoing such treatment. Alternatively, in some circumstances, it may be desirable to provide multiple, or successive administrations of the AAV vector compositions, either over a relatively short, or a relatively prolonged period of time, as may be determined by the medical practitioner overseeing the administration of such compositions. For example, the number of infectious particles administered to a mammal may be on the order of about 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13}, or even higher, infectious particles/ml given either as a single dose, or divided into two or more administrations as may be required to achieve therapy of the particular disease or disorder being treated. In fact, in certain embodiments, it may be desirable to administer two or more different AAV vector compositions, either alone, or in combination with one or more other neurotherapeutic drugs to achieve the desired effects of a particular therapy regimen.

4.2 LIPOSOME-, NANOCAPSULE-, AND MICROPARTICLE-MEDIATED DELIVERY

In certain embodiments, the inventors contemplate the use of liposomes, nanocapsules, microparticles, microspheres, lipid particles, vesicles, and the like, for the introduction of the compositions of the present invention into suitable host cells. In particular, the rAAV vector delivered gene therapy compositions of the present invention may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like.

Such formulations may be preferred for the introduction of pharmaceutically acceptable formulations of the nucleic acids or the rAAV constructs disclosed herein. The formation and use of liposomes is generally known to those of skill in the art (see for example, Couvreur *et al.*, 1977; Couvreur, 1988; Lasic, 1998; which describes the use of liposomes and nanocapsules in the targeted antibiotic therapy for intracellular bacterial infections and diseases). Recently, liposomes were developed with improved serum stability and circulation half-times (Gabizon and Papahadjopoulos, 1988; Allen and Choun, 1987; U. S. Patent 5,741,516, specifically incorporated herein by reference in its entirety). Further, various methods of liposome and liposome like preparations as potential drug carriers have been reviewed (Takakura, 1998; Chandran *et al.*, 1997; Margalit, 1995; U. S. Patent 5,567,434; U. S. Patent 5,552,157; U. S. Patent 5,565,213; U. S. Patent 5,738,868 and U. S. Patent 5,795,587, each specifically incorporated herein by reference in its entirety).

Liposomes have been used successfully with a number of cell types that are normally resistant to transfection by other procedures including T cell suspensions, primary hepatocyte cultures and PC 12 cells (Renneisen *et al.*, 1990; Muller *et al.*, 1990). In addition, liposomes are free of the DNA length constraints that are typical of viral-based delivery systems. Liposomes have been used effectively to introduce genes, drugs (Heath and Martin, 1986; Heath *et al.*, 1986; Balazsovits *et al.*, 1989; Fresta and Puglisi, 1996), radiotherapeutic agents (Pikul *et al.*, 1987), enzymes (Imaizumi *et al.*, 1990a; Imaizumi *et al.*, 1990b), viruses (Faller and Baltimore, 1984), transcription factors and allosteric effectors (Nicolau and Gersonde, 1979) into a variety of cultured cell lines and animals. In addition, several successful clinical trails examining the effectiveness of liposome-mediated drug delivery have been completed (Lopez-Berestein *et al.*, 1985a; 1985b; Coune, 1988; Sculier *et al.*, 1988). Furthermore, several studies suggest that the use of liposomes is not associated with autoimmune responses, toxicity or gonadal localization after systemic delivery (Mori and Fukatsu, 1992).

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs). MLVs generally have diameters of from 25 nm to 4 μm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core.

Liposomes bear resemblance to cellular membranes and are contemplated for use in connection with the present invention as carriers for the peptide compositions. They are widely suitable as both water- and lipid-soluble substances can be entrapped, *i.e.* in the aqueous spaces and within the bilayer itself, respectively. It is possible that the drug-bearing liposomes may even be employed for site-specific delivery of active agents by selectively modifying the liposomal formulation.

In addition to the teachings of Couvreur *et al.* (1977; 1988), the following information may be utilized in generating liposomal formulations. Phospholipids can form a variety of structures other than liposomes when dispersed in water, depending on the molar ratio of lipid to water. At low ratios the liposome is the preferred structure. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

In addition to temperature, exposure to proteins can alter the permeability of liposomes. Certain soluble proteins, such as cytochrome c, bind, deform and penetrate the bilayer, thereby causing changes in permeability. Cholesterol inhibits this penetration of proteins, apparently by packing the phospholipids more tightly. It is contemplated that the most useful liposome formations for antibiotic and inhibitor delivery will contain cholesterol.

The ability to trap solutes varies between different types of liposomes. For example, MLVs are moderately efficient at trapping solutes, but SUVs are extremely inefficient. SUVs offer the advantage of homogeneity and reproducibility in size distribution, however, and a compromise between size and trapping efficiency is offered by large unilamellar vesicles (LUVs). These are prepared by ether evaporation and are three to four times more efficient at solute entrapment than MLVs.

In addition to liposome characteristics, an important determinant in entrapping compounds is the physicochemical properties of the compound itself. Polar compounds are

trapped in the aqueous spaces and nonpolar compounds bind to the lipid bilayer of the vesicle. Polar compounds are released through permeation or when the bilayer is broken, but nonpolar compounds remain affiliated with the bilayer unless it is disrupted by temperature or exposure to lipoproteins. Both types show maximum efflux rates at the phase transition temperature.

Liposomes interact with cells *via* four different mechanisms: Endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or vice versa, without any association of the liposome contents. It often is difficult to determine which mechanism is operative and more than one may operate at the same time.

The fate and disposition of intravenously injected liposomes depend on their physical properties, such as size, fluidity, and surface charge. They may persist in tissues for h or days, depending on their composition, and half lives in the blood range from min to several h. Larger liposomes, such as MLVs and LUVs, are taken up rapidly by phagocytic cells of the reticuloendothelial system, but physiology of the circulatory system restrains the exit of such large species at most sites. They can exit only in places where large openings or pores exist in the capillary endothelium, such as the sinusoids of the liver or spleen. Thus, these organs are the predominate site of uptake. On the other hand, SUVs show a broader tissue distribution but still are sequestered highly in the liver and spleen. In general, this *in vivo* behavior limits the potential targeting of liposomes to only those organs and tissues accessible to their large size. These include the blood, liver, spleen, bone marrow, and lymphoid organs.

Targeting is generally not a limitation in terms of the present invention. However, should specific targeting be desired, methods are available for this to be accomplished. Antibodies may be used to bind to the liposome surface and to direct the antibody and its drug contents to specific antigenic receptors located on a particular cell-type surface. Carbohydrate determinants (glycoprotein or glycolipid cell-surface components that play a role in cell-cell recognition, interaction and adhesion) may also be used as recognition sites as they have potential in directing liposomes to particular cell types. Mostly, it is contemplated that intravenous injection of liposomal preparations would be used, but other routes of administration are also conceivable.

Alternatively, the invention provides for pharmaceutically acceptable nanocapsule formulations of the AAV vector-based polynucleotide compositions of the present invention. Nanocapsules can generally entrap compounds in a stable and reproducible way (Henry-Michelland *et al.*, 1987; Quintanar-Guerrero *et al.*, 1998; Douglas *et al.*, 1987). To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention. Such particles may be are easily made, as described (Couvreur *et al.*, 1980; Couvreur, 1988; zur Muhlen *et al.*, 1998; Zambaux *et al.* 1998; Pinto-Alphandry *et al.*, 1995 and U. S. Patent 5,145,684, specifically incorporated herein by reference in its entirety).

4.3 Additional Modes of Delivery

In addition to the methods of delivery described above, the following techniques are also contemplated as alternative methods of delivering the disclosed rAAV vector based polynucleotide compositions to a target cell or animal. Sonophoresis (*i.e.*, ultrasound) has been used and described in U. S. Patent 5,656,016 (specifically incorporated herein by reference in its entirety) as a device for enhancing the rate and efficacy of drug permeation into and through the circulatory system. Other drug delivery alternatives contemplated are intraosseous injection (U. S. Patent 5,779,708), microchip devices (U. S. Patent 5,797,898), ophthalmic formulations (Bourlais *et al.*, 1998), transdermal matrices (U. S. Patent 5,770,219 and U. S. Patent 5,783,208) and feedback-controlled delivery (U. S. Patent 5,697,899), each specifically incorporated herein by reference in its entirety.

4.4 AAV Biology and Recombinant AAV (rAAV) Vectors for Gene Transfer

Adeno-associated virus (AAV) is a single-stranded DNA parvovirus with a 4.7 kb genome and a particle diameter of approximately 20 nm. The AAV genome is flanked by two identical inverted terminal repeat (ITR) sequences (Lusby *et al.*, 1980). These ITRs provide all the *cis*-acting sequence required for replication, packaging and integration (Samulski *et al.*, 1989). There are two large open reading frames (Srivastava *et al.*, 1983). The open reading frame in the right half of the genome (*cap*) encodes 3 overlapping coat proteins (VP1, VP2 and VP3). The open reading frame in the left half (*rep* gene) encodes 4 regulatory proteins with overlapping sequences which are known as *Rep* proteins (*Rep*78, *Rep*68, *Rep*52 and *Rep*40), because frame shift mutations at most locations within the open reading frame inhibit viral DNA replication (Hermonat *et al.*, 1984). The *Rep* proteins are multi-functional DNA binding

proteins. The functions of the *Rep* proteins in viral DNA replication include helicase activity and a site-specific, strand-specific endonuclease (nicking) activity (Ni *et al.*, 1994).

AAV infects a broad spectrum of vertebrates from birds to humans, although in nature specific types are species specific (Berns, 1996). In humans AAV can infect a large variety of cells derived from different tissues. The infection of AAV is ubiquitous within the population with about 90% of adults being seropositive (Cukor *et al.*, 1983). In spite of its omnipresence, AAV has never been associated with any human disease. In this sense, rAAV is the safest of the currently used gene therapy vectors.

Because of its propensity to establish latency and because it has not been implicated as a pathogen, AAV has been of considerable interest as a potential vector for human gene therapy (Flotte and Ferkol, 1997; Flotte and Carter, 1995). In general, rAAV vectors are produced by deleting the viral coding sequences and substituting the transgene of interest under control of a non-AAV promoter between the two AAV inverted terminal repeats (ITRs). When the *rep* and *cap* proteins are expressed *in trans* in Ad-infected cells, rAAV genomes can be efficiently packaged. Considerations in the development of AAV as a vector have included difficulties in attaining high vector titers and the limited insertional capacity (>5 kb). Although these issues can still be improved, recently developed packaging techniques for high titer and Ad-contamination free vectors, and strategies to overcome the packaging limitation, have dramatically impacted the applications of rAAV (Zolotukhin *et al.*, 1999; Duan *et al.*, 2000; Yan *et al.*, 2000). Unlike adenovirus vectors, rAAV vectors are remarkably nonimmunogenic with little host response (Jooss *et al.*, 1998; Song *et al.*, 1998). In addition to the above unique features, rAAV have mediated long-term transgene expression in a wide variety of tissues, including muscle (Song *et al.*, 1998; Kessler *et al.*, 1996; Xiao *et al.*, 1996; Clark *et al.*, 1997; Snyder *et al.*, 1997a), lung (Flotte *et al.*, 1993), liver (Snyder *et al.*, 1997b; Xiao *et al.*, 1998; Song *et al.*, 2001a; Xu *et al.*, 2001), brain (Kaplitt *et al.*, 1994) and eye (Flannery *et al.*, 1997). Thus rAAV vectors appear to have significant advantages over other commonly used viral vectors.

Six serotypes of AAV have been cloned and sequenced. Of the six AAV serotypes, serotype 2 (AAV2) is the best-characterized and has been predominantly used in gene transfer studies. Membrane-associated heparan sulfate proteoglycan is the primary receptor for AAV type 2 (Summerford and Samulski, 1998). Human fibroblast growth factor receptor 1 and $\alpha_v\beta_5$ integrin are co-receptors for AAV2 (Qing *et al.*, 1999; Summerford *et al.*, 1999). Serotypes 1 and 6 share >99% amino acid homology in their capsid proteins. Sequence analysis supports a recombination event between serotype I and 2. Comparison of the serotype capsid amino acid

sequences suggests that serotypes, 1, 2, and 3 share homology across the three capsids in accord with heparan sulfate binding (Summerford and Samulski, 1998). In contrast, AAV type 4 and 5 are the most divergent of the six AAV serotypes, exhibiting only 60% homology to AAV2 or to each other. AAV4 and AAV5 require different sialic acid-containing glycoproteins for binding and transduction of target cells. The different tropisms of AAV serotypes provide opportunities to optimize the transduction efficiency in different target cells. Data showed that of the serotypes, AAV1 mediated the highest transgene expression in skeletal muscle and murine islets (Chao *et al.*, 2000).

4.5 PROMOTERS AND ENHANCERS

Recombinant AAV vectors form important aspects of the present invention. The term "expression vector or construct" means any type of genetic construct containing a nucleic acid in which part or all of the nucleic acid encoding sequence is capable of being transcribed. In preferred embodiments, expression only includes transcription of the nucleic acid, for example, to generate a biologically-active peptide, protein, polypeptide, antisense molecule, or catalytic RNA ribozyme from a transcribed gene.

Particularly useful vectors are contemplated to be those vectors in which the nucleic acid segment to be transcribed is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively positioned," "under control" or "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

In preferred embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with an cytokine or serpin-encoding gene in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters isolated from any bacterial, viral, eukaryotic, or mammalian cell.

Naturally, it will be important to employ a promoter that effectively directs the expression of the serpin or cytokine-encoding DNA segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be

constitutive, or inducible, and can be used under the appropriate conditions to direct high-level expression of the introduced DNA segment, or the promoters may direct tissue- or cell-specific expression of the therapeutic constructs, such as, for example, an islet cell- or pancreas-specific promoter such as the insulin promoter.

At least one module in a promoter functions to position the start site for RNA synthesis. The best-known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid is not believed to be critical, so long as it is capable of expressing the serpin or cytokine-polypeptide encoding nucleic acid segment in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter, such as a CMV or an HSV promoter. In certain aspects of the invention, β-actin, and in particular, chicken β-actin promoters have been shown to be particularly preferred for certain embodiments of the invention.

In various other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of transgenes. The use of other viral or mammalian cellular or bacterial phage promoters that are well known in the art to achieve expression of a transgene is contemplated as well, provided that the levels of expression are sufficient for a given purpose. A variety of promoter elements have been described in Tables 1 and 2 that may be employed, in the context of the present invention, to regulate the expression of the

present serpin or cytokine-encoding nucleic acid segments comprised within the recombinant AAV vectors of the present invention.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 1

ILLUSTRATIVE PROMOTER AND ENHANCER ELEMENTS

PROMOTER/ENHANCER	REFERENCES
Immunoglobulin Heavy Chain	Banerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1984; Kiledjian *et al.*, 1988; Porton *et al.*; 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria *et al.*, 1987; Winoto and Baltimore, 1989; Redondo *et al.*; 1990
HLA DQ a and DQ β	Sullivan and Peterlin, 1987
β-Interferon	Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn and Maniatis, 1988

PROMOTER/ENHANCER	REFERENCES
Interleukin-2	Greene *et al.*, 1989
Interleukin-2 Receptor	Greene *et al.*, 1989; Lin *et al.*, 1990
MHC Class II 5	Koch *et al.*, 1989
MHC Class II HLA-Dra	Sherman *et al.*, 1989
β-Actin	Kawamoto *et al.*, 1988; Ng *et al.*; 1989
Muscle Creatine Kinase	Jaynes *et al.*, 1988; Horlick and Benfield, 1989; Johnson *et al.*, 1989
Prealbumin (Transthyretin)	Costa *et al.*, 1988
Elastase I	Omitz *et al.*, 1987
Metallothionein	Karin *et al.*, 1987; Culotta and Hamer, 1989
Collagenase	Pinkert *et al.*, 1987; Angel *et al.*, 1987
Albumin Gene	Pinkert *et al.*, 1987; Tronche *et al.*, 1989, 1990
α-Fetoprotein	Godbout *et al.*, 1988; Campere and Tilghman, 1989
t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
β-Globin	Trudel and Constantini, 1987
e-fos	Cohen *et al.*, 1987
c-HA-ras	Triesman, 1986; Deschamps *et al.*, 1985
Insulin	Edlund *et al.*, 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh *et al.*, 1990
$\alpha_{1\text{-Antitrypain}}$	Latimer *et al.*, 1990
H2B (TH2B) Histone	Hwang *et al.*, 1990
Mouse or Type I Collagen	Ripe *et al.*, 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang *et al.*, 1989
Rat Growth Hormone	Larsen *et al.*, 1986
Human Serum Amyloid A (SAA)	Edbrooke *et al.*, 1989
Troponin I (TN I)	Yutzey *et al.*, 1989
Platelet-Derived Growth Factor	Pech *et al.*, 1989
Duchenne Muscular Dystrophy	Klamut *et al.*, 1990
SV40	Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987; Schaffner *et al.*, 1988

PROMOTER/ENHANCER	REFERENCES
Polyoma	Swartzendruber and Lehman, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; de Villiers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and Villarreal, 1988
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a, b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander and Haseltine, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Chol *et al.*, 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and Wilkie, 1983; Spalholz *et al.*, 1985; Lusky and Botchan, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987; Stephens and Hentschel, 1987
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988; Vannice and Levinson, 1988
Human Immunodeficiency Virus	Muesing *et al.*, 1987; Hauber and Cullan, 1988; Jakobovits *et al.*, 1988; Feng and Holland, 1988; Takebe *et al.*, 1988; Rosen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp and Marciniak, 1989; Braddock *et al.*, 1989
Cytomegalovirus	Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook *et al.*, 1987; Quinn *et al.*, 1989

TABLE 2

INDUCIBLE ELEMENTS

ELEMENT	INDUCER	REFERENCES
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter *et al.*, 1982; Haslinger and Karin, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987, Karin *et al.*, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang *et al.*, 1981; Lee *et al.*, 1981; Majors and Varmus, 1983;

ELEMENT	INDUCER	REFERENCES
		Chandler *et al.*, 1983; Lee *et al.*, 1984; Ponta *et al.*, 1985; Sakai *et al.*, 1988
β-Interferon	poly(rI)x poly(rc)	Tavernier *et al.*, 1983
Adenovirus 5 E2	Ela	Imperiale and Nevins, 1984
Collagenase	Phorbol Ester (TPA)	Angel *et al.*, 1987a
Stromelysin	Phorbol Ester (TPA)	Angel *et al.*, 1987b
SV40	Phorbol Ester (TPA)	Angel *et al.*, 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	
GRP78 Gene	A23187	Resendez *et al.*, 1988
α-2-Macroglobulin	IL-6	Kunz *et al.*, 1989
Vimentin	Serum	Rittling *et al.*, 1989
MHC Class I Gene H-2κb	Interferon	Blanar *et al.*, 1989
HSP70	Ela, SV40 Large T Antigen	Taylor *et al.*, 1989; Taylor and Kingston, 1990a, b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	FMA	Hensel *et al.*, 1989
Thyroid Stimulating Hormone a Gene	Thyroid Hormone	Chatterjee *et al.*, 1989

As used herein, the terms "engineered" and "recombinant" cells are intended to refer to a cell into which an exogenous DNA segment, such as DNA segment that leads to the transcription of a biologically-active serpin or cytokine polypeptide or a ribozyme specific for such a biologically-active serpin or cytokine polypeptide product, has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells, which do not contain a recombinantly introduced exogenous DNA segment. Engineered cells are thus cells having DNA segment introduced through the hand of man.

To express a biologically-active serpin or cytokine encoding gene in accordance with the present invention one would prepare an rAAV expression vector that comprises a biologically-active serpin or cytokine polypeptide-encoding nucleic acid segment under the control of one or more promoters. To bring a sequence "under the control of" a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame generally between

about 1 and about 50 nucleotides "downstream" of (*i.e.*, 3' of) the chosen promoter. The "upstream" promoter stimulates transcription of the DNA and promotes expression of the encoded polypeptide. This is the meaning of "recombinant expression" in this context. Particularly preferred recombinant vector constructs are those that comprise an rAAV vector. Such vectors are described in detail herein.

4.6 Mutagenesis and Preparation of Modified Nucleotide Compositions

In certain embodiments, it may be desirable to prepared modified nucleotide compositions, such as, for example, in the generation of the nucleic acid segments that encode either parts of the AAV vector itself, or the promoter, or even the therapeutic gene delivered by such rAAV vectors. Various means exist in the art, and are routinely employed by the artisan to generate modified nucleotide compositions.

Site-specific mutagenesis is a technique useful in the preparation and testing of sequence variants by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art. As will be appreciated, the technique typically employs a bacteriophage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector, or melting of two strands of a double stranded vector that includes within its sequence a DNA sequence encoding the desired ribozyme or other nucleic acid construct. An oligonucleotide primer bearing the desired mutated sequence is synthetically prepared. This primer is then annealed with the single-stranded DNA preparation, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand

encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected nucleic acid sequences using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting, as there are other ways in which sequence variants may be obtained. For example, recombinant vectors encoding the desired gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

4.7 NUCLEIC ACID AMPLIFICATION

In certain embodiments, it may be necessary to employ one or more nucleic acid amplification techniques to produce the nucleic acid segments of the present invention. Various methods are well-known to artisans in the field, including for example, those techniques described herein:

Nucleic acid, used as a template for amplification, may be isolated from cells contained in the biological sample according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA and is used directly as the template for amplification.

Pairs of primers that selectively hybridize to nucleic acids corresponding to the ribozymes or conserved flanking regions are contacted with the isolated nucleic acid under conditions that permit selective hybridization. The term "primer", as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred.

Once hybridized, the nucleic acid:primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

Next, the amplification product is detected. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or

fluorescent label or even *via* a system using electrical or thermal impulse signals (*e.g.*, Affymax technology).

A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best-known amplification methods is the polymerase chain reaction (referred to as PCR™), which is described in detail in U. S. Patent No. 4,683,195, U. S. Patent No. 4,683,202 and U. S. Patent No. 4,800,159 (each of which is incorporated herein by reference in its entirety).

Briefly, in PCR™, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates is added to a reaction mixture along with a DNA polymerase, *e.g.*, *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated.

A reverse transcriptase PCR™ amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.* (1989). Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases. These methods are described in Int. Pat. Appl. Publ. No. WO 90/07641 (specifically incorporated herein by reference). Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPA No. 320 308, and incorporated herein by reference in its entirety. In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR™, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

Qβ Replicase (QβR), described in Int. Pat. Appl. No. PCT/US87/00880, incorporated herein by reference, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to

that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[α-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention.

Strand Displacement Amplification (SDA), described in U. S. Patent Nos. 5,455,166, 5,648,211, 5,712,124 and 5,744,311, each incorporated herein by reference, is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

Still another amplification methods described in GB Application No. 2 202 328, and in Int. Pat. Appl. No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR™-like, template- and enzyme-dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes is added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR Gingeras *et al.*, Int. Pat. Appl. Publ. No. WO 88/10315, incorporated herein by reference. In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These

amplification techniques involve annealing a primer that has target specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat denatured again. In either case the single stranded DNA is made fully double stranded by addition of second target specific primer, followed by polymerization. The double-stranded DNA molecules are then multiply transcribed by an RNA polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNAs are reverse transcribed into single stranded DNA, which is then converted to double stranded DNA, and then transcribed once again with an RNA polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate target specific sequences.

Davey *et al.*, EPA No. 329 822 (incorporated herein by reference in its entirety) disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

Miller *et al.*, Int. Pat. Appl. Publ. No. WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR™" (Frohman, 1990, specifically incorporated herein by reference).

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide," thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention.

Following any amplification, it may be desirable to separate the amplification product from the template and the excess primer for the purpose of determining whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods (see *e.g.,* Sambrook *et al.*, 1989).

Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography.

Amplification products must be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled, nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art and can be found in many standard books on molecular protocols. See Sambrook *et al.*, 1989. Briefly, amplification products are separated by gel electrophoresis. The gel is then contacted with a membrane, such as nitrocellulose, permitting transfer of the nucleic acid and non-covalent binding. Subsequently, the membrane is incubated with a chromophore-conjugated probe that is capable of hybridizing with a target amplification product. Detection is by exposure of the membrane to x-ray film or ion-emitting detection devices.

One example of the foregoing is described in U. S. Patent No. 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and

blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

4.8 METHODS OF NUCLEIC ACID DELIVERY AND DNA TRANSFECTION

In certain embodiments, it is contemplated that one or more RNA, DNA, PNAs and/or substituted polynucleotide compositions disclosed herein will be used to transfect an appropriate host cell. Technology for introduction of PNAs, RNAs, and DNAs into cells is well known to those of skill in the art.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Wong and Neumann, 1982; Fromm *et al.*, 1985; Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984; Suzuki et al., 1998; Vanbever *et al.*, 1998), direct microinjection (Capecchi, 1980; Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Takakura, 1998) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990; Klein *et al.*, 1992), and receptor-mediated transfection (Curiel *et al.*, 1991; Wagner *et al.*, 1992; Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

4.9 EXPRESSION VECTORS

The present invention contemplates a variety of AAV-based expression systems, and vectors. In one embodiment the preferred AAV expression vectors comprise at least a first nucleic acid segment that encodes a therapeutic antisense molecule. In another embodiment, a promoter is operatively linked to a sequence region that encodes a functional mRNA, a tRNA, a ribozyme or an antisense RNA.

As used herein, the term "operatively linked" means that a promoter is connected to a functional RNA in such a way that the transcription of that functional RNA is controlled and regulated by that promoter. Means for operatively linking a promoter to a functional RNA are well known in the art.

The choice of which expression vector and ultimately to which promoter a polypeptide coding region is operatively linked depend directly on the functional properties desired, *e.g.*, the location and timing of protein expression, and the host cell to be transformed. These are well known limitations inherent in the art of constructing recombinant DNA molecules. However, a

vector useful in practicing the present invention is capable of directing the expression of the functional RNA to which it is operatively linked.

RNA polymerase transcribes a coding DNA sequence through a site where polyadenylation occurs. Typically, DNA sequences located a few hundred base pairs downstream of the polyadenylation site serve to terminate transcription. Those DNA sequences are referred to herein as transcription-termination regions. Those regions are required for efficient polyadenylation of transcribed messenger RNA (mRNA).

A variety of methods have been developed to operatively link DNA to vectors *via* complementary cohesive termini or blunt ends. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted and to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

4.10 BIOLOGICAL FUNCTIONAL EQUIVALENTS

Modification and changes to the structure of the polynucleotides and polypeptides of wild-type rAAV vectors to provide the improved rAAV virions as described in the present invention to obtain functional viral vectors that possess desirable characteristics, particularly with respect to improved delivery of therapeutic gene constructs to selected mammalian cell, tissues, and organs for the treatment, prevention, and prophylaxis of various diseases and disorders, as well as means for the amelioration of symptoms of such diseases, and to facilitate the expression of exogenous therapeutic and/or prophylactic polypeptides of interest *via* rAAV vector-mediated gene therapy. As mentioned above, one of the key aspects of the present invention is the creation of one or more mutations into specific polynucleotide sequences that encode one or more of the therapeutic agents encoded by the disclosed rAAV constructs. In certain circumstances, the resulting polypeptide sequence is altered by these mutations, or in other cases, the sequence of the polypeptide is unchanged by one or more mutations in the encoding polynucleotide to produce modified vectors with improved properties for effecting gene therapy in mammalian systems.

When it is desirable to alter the amino acid sequence of a polypeptide to create an equivalent, or even an improved, second-generation molecule, the amino acid changes may be achieved by changing one or more of the codons of the encoding DNA sequence, according to Table 3.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for

example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the polynucleotide sequences disclosed herein, without appreciable loss of their biological utility or activity.

TABLE 3

Amino Acids		Codons						
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a

protein is generally understood in the art (Kyte and Doolittle, 1982, incorporate herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (–0.4); threonine (–0.7); serine (-0.8); tryptophan (–0.9); tyrosine (–1.3); proline (–1.6); histidine (–3.2); glutamate (-3.5); glutamine (–3.5); aspartate (–3.5); asparagine (–3.5); lysine (–3.9); and arginine (–4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.* still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those that are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U. S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U. S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (–0.4); proline (–0.5 ± 1); alanine (–0.5); histidine (–0.5); cysteine (-1.0); methionine (–1.3); valine (–1.5); leucine (–1.8); isoleucine (–1.8); tyrosine (-2.3); phenylalanine (–2.5); tryptophan (–3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those that are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take several of the foregoing characteristics into consideration are well known to those of skill in the art and

include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

4.11 THERAPEUTIC AND DIAGNOSTIC KITS

The invention also encompasses one or more rAAV vector compositions together with one or more pharmaceutically-acceptable excipients, carriers, diluents, adjuvants, and/or other components, as may be employed in the formulation of particular rAAV-polynucleotide delivery formulations, and in the preparation of therapeutic agents for administration to a mammal, and in particularly, to a human. In particular, such kits may comprise one or more of the disclosed rAAV compositions in combination with instructions for using the viral vector in the treatment of such disorders in a mammal, and may typically further include containers prepared for convenient commercial packaging.

As such, preferred animals for administration of the pharmaceutical compositions disclosed herein include mammals, and particularly humans. Other preferred animals include murines, bovines, equines, porcines, canines, and felines. The composition may include partially or significantly purified rAAV compositions, either alone, or in combination with one or more additional active ingredients, which may be obtained from natural or recombinant sources, or which may be obtainable naturally or either chemically synthesized, or alternatively produced *in vitro* from recombinant host cells expressing DNA segments encoding such additional active ingredients.

Therapeutic kits may also be prepared that comprise at least one of the compositions disclosed herein and instructions for using the composition as a therapeutic agent. The container means for such kits may typically comprise at least one vial, test tube, flask, bottle, syringe or other container means, into which the disclosed rAAV composition(s) may be placed, and preferably suitably aliquoted. Where a second therapeutic polypeptide composition is also provided, the kit may also contain a second distinct container means into which this second composition may be placed. Alternatively, the plurality of therapeutic biologically active compositions may be prepared in a single pharmaceutical composition, and may be packaged in a single container means, such as a vial, flask, syringe, bottle, or other suitable single container means. The kits of the present invention will also typically include a means for containing the vial(s) in close confinement for commercial sale, such as, *e.g.*, injection or blow-molded plastic containers into which the desired vial(s) are retained.

4.12 RIBOZYMES

Another aspect of the invention concerns the use of AAV1 and AAV5 serotype vectors to deliver ribozymes to selected neural cells and tissues. Ribozymes are biological catalysts consisting of only RNA. They promote a variety of reactions involving RNA and DNA molecules including site-specific cleavage, ligation, polymerization, and phosphoryl exchange (Cech, 1989; Cech, 1990). Ribozymes fall into three broad classes: (1) RNAse P, (2) self-splicing introns, and (3) self-cleaving viral agents. Self-cleaving agents include hepatitis delta virus and components of plant virus satellite RNAs that sever the RNA genome as part of a rolling-circle mode of replication. Because of their small size and great specificity, ribozymes have the greatest potential for biotechnical applications. The ability of ribozymes to cleave other RNA molecules at specific sites in a catalytic manner has brought them into consideration as inhibitors of viral replication or of cell proliferation and gives them potential advantage over antisense RNA. Indeed, ribozymes have already been used to cleave viral targets and oncogene products in living cells (Koizumi *et al.*, 1992; Kashani-Sabet *et al.*, 1992; Taylor and Rossi, 1991; von-Weizsacker *et al.*, 1992; Ojwang *et al.*, 1992; Stephenson and Gibson, 1991; Yu *et al.*, 1993; Xing and Whitton, 1993; Yu *et al.*, 1995; Little and Lee, 1995).

Two kinds of ribozymes have been employed widely, hairpins and hammerheads. Both catalyze sequence-specific cleavage resulting in products with a 5N hydroxyl and a 2N,3N-cyclic phosphate. Hammerhead ribozymes have been used more commonly, because they impose few restrictions on the target site. Hairpin ribozymes are more stable and, consequently, function better than hammerheads at physiologic temperature and magnesium concentrations.

A number of patents have issued describing various ribozymes and methods for designing ribozymes. See, for example, U.S. Patent Nos. 5,646,031; 5,646,020; 5,639,655; 5,093,246; 4,987,071; 5,116,742; and 5,037,746, each specifically incorporated herein by reference in its entirety. However, the ability of ribozymes to provide therapeutic benefit *in vivo* has not yet been demonstrated.

Although proteins traditionally have been used for catalysis of nucleic acids, another class of macromolecules has emerged as useful in this endeavor. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, 1987; Gerlach *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to

the requirement that the substrate bind *via* specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence-specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cech *et al.*, 1981). For example, U. S. Patent No. 5,354,855 (specifically incorporated herein by reference) reports that certain ribozymes can act as endonucleases with a sequence-specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to neurotherapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990). Recently, it was reported that ribozymes elicited genetic changes in some cells lines to which they were applied; the altered genes included the oncogenes H-*ras*, c-*fos* and genes of HIV. Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme.

Six basic varieties of naturally occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nature of a ribozyme is advantageous over many technologies, such as antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the concentration of ribozyme necessary to affect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can completely eliminate catalytic activity of a ribozyme. Similar mismatches in antisense molecules do not prevent their action (Woolf *et al.*, 1992).

Thus, the specificity of action of a ribozyme is greater than that of an antisense oligonucleotide binding the same RNA site.

The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, a hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence) or Neurospora VS RNA motif. Examples of hammerhead motifs are described by Rossi *et al.* (1992). Examples of hairpin motifs are described by Hampel *et al.* (Eur. Pat. Appl. Publ. No. EP 0360257), Hampel and Tritz (1989), Hampel *et al.* (1990) and U. S. Patent 5,631,359 (specifically incorporated herein by reference). An example of the hepatitis δ virus motif is described by Perrotta and Been (1992); an example of the RNaseP motif is described by Guerrier-Takada *et al.* (1983); Neurospora VS RNA ribozyme motif is described by Collins (Saville and Collins, 1990; Saville and Collins, 1991; Collins and Olive, 1993); and an example of the Group I intron is described in U. S. Patent 4,987,071 (specifically incorporated herein by reference). All that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus the ribozyme constructs need not be limited to specific motifs mentioned herein.

In certain embodiments, it may be important to produce enzymatic cleaving agents that exhibit a high degree of specificity for the RNA of a desired target, such as one of the sequences disclosed herein. The enzymatic nucleic acid molecule is preferably targeted to a highly conserved sequence region of a target mRNA. Such enzymatic nucleic acid molecules can be delivered exogenously to specific cells as required, although in preferred embodiments the ribozymes are expressed from DNA or RNA vectors that are delivered to specific cells.

Small enzymatic nucleic acid motifs (*e.g.*, of the hammerhead or the hairpin structure) may also be used for exogenous delivery. The simple structure of these molecules increases the ability of the enzymatic nucleic acid to invade targeted regions of the mRNA structure. Alternatively, catalytic RNA molecules can be expressed within cells from eukaryotic promoters (*e.g.*, Scanlon *et al.*, 1991; Kashani-Sabet *et al.*, 1992; Dropulic *et al.*, 1992; Weerasinghe *et al.*, 1991; Ojwang *et al.*, 1992; Chen *et al.*, 1992; Sarver *et al.*, 1990). Those skilled in the art realize that any ribozyme can be expressed in eukaryotic cells from the appropriate DNA vector. The activity of such ribozymes can be augmented by their release from the primary transcript by a second ribozyme (Int. Pat. Appl. Publ. No. WO 93/23569, and Int. Pat. Appl. Publ. No. WO 94/02595, both hereby incorporated by reference; Ohkawa *et al.*, 1992; Taira *et al.*, 1991; and Ventura *et al.*, 1993).

Ribozymes may be added directly, or can be complexed with cationic lipids, lipid complexes, packaged within liposomes, or otherwise delivered to target cells. The RNA or RNA complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through injection, aerosol inhalation, infusion pump or stent, with or without their incorporation in biopolymers.

Ribozymes may be designed as described in Int. Pat. Appl. Publ. No. WO 93/23569 and Int. Pat. Appl. Publ. No. WO 94/02595 (each specifically incorporated herein by reference) and synthesized to be tested *in vitro* and *in vivo*, as described. Such ribozymes can also be optimized for delivery. While specific examples are provided, those in the art will recognize that equivalent RNA targets in other species can be utilized when necessary.

Hammerhead or hairpin ribozymes may be individually analyzed by computer folding (Jaeger *et al.*, 1989) to assess whether the ribozyme sequences fold into the appropriate secondary structure, as described herein. Those ribozymes with unfavorable intramolecular interactions between the binding arms and the catalytic core are eliminated from consideration. Varying binding arm lengths can be chosen to optimize activity. Generally, at least 5 or so bases on each arm are able to bind to, or otherwise interact with, the target RNA.

Ribozymes of the hammerhead or hairpin motif may be designed to anneal to various sites in the mRNA message, and can be chemically synthesized. The method of synthesis used follows the procedure for normal RNA synthesis as described in Usman *et al.* (1987) and in Scaringe *et al.* (1990) and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5′-end, and phosphoramidites at the 3′-end. Average stepwise coupling yields are typically >98%. Hairpin ribozymes may be synthesized in two parts and annealed to reconstruct an active ribozyme (Chowrira and Burke, 1992). Ribozymes may be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2′-amino, 2′-C-allyl, 2′-flouro, 2′-o-methyl, 2′-H (for a review see *e.g.*, Usman and Cedergren, 1992). Ribozymes may be purified by gel electrophoresis using general methods or by high-pressure liquid chromatography and resuspended in water.

Ribozyme activity can be optimized by altering the length of the ribozyme binding arms, or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (see *e.g.*, Int. Pat. Appl. Publ. No. WO 92/07065; Perrault *et al*, 1990; Pieken *et al.*, 1991; Usman and Cedergren, 1992; Int. Pat. Appl. Publ. No. WO 93/15187; Int. Pat. Appl. Publ. No. WO 91/03162; Eur. Pat. Appl. Publ. No. 92110298.4; U.S. Patent 5,334,711; and Int. Pat. Appl. Publ. No. WO 94/13688, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules),

modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

A preferred means of accumulating high concentrations of a ribozyme(s) within cells is to incorporate the ribozyme-encoding sequences into a DNA expression vector. Transcription of the ribozyme sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, *etc.*) present nearby. Prokaryotic RNA polymerase promoters may also be used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990; Gao and Huang, 1993; Lieber *et al.*, 1993; Zhou *et al.*, 1990). Ribozymes expressed from such promoters can function in mammalian cells (Kashani-Sabet *et al.*, 1992; Ojwang *et al.*, 1992; Chen *et al.*, 1992; Yu *et al.*, 1993; L'Huillier *et al.*, 1992; Lisziewicz *et al.*, 1993). Although incorporation of the present ribozyme constructs into adeno-associated viral vectors is preferred, such transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, other viral DNA vectors (such as adenovirus vectors), or viral RNA vectors (such as retroviral, semliki forest virus, sindbis virus vectors).

Sullivan *et al.* (Int. Pat. Appl. Publ. No. WO 94/02595) describes general methods for delivery of enzymatic RNA molecules. Ribozymes may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. For some indications, ribozymes may be directly delivered *ex vivo* to cells or tissues with or without the aforementioned vehicles. Alternatively, the RNA/vehicle combination may be locally delivered by direct inhalation, by direct injection or by use of a catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular, intramuscular, subcutaneous or joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraocular, retinal, subretinal, intraperitoneal, intracerebroventricular, intrathecal delivery, and/or direct injection to one or more tissues of the brain. More detailed descriptions of ribozyme and rAAV vector delivery and administration are provided in Int. Pat. Appl. Publ. No. WO 94/02595 and Int. Pat. Appl. Publ. No. WO 93/23569, each specifically incorporated herein by reference.

Ribozymes and the AAV vectored-constructs of the present invention may be used to inhibit gene expression and define the role (essentially) of specified gene products in the

progression of one or more neural diseases, dysfunctions, cancers,, and/or disorders. In this manner, other genetic targets may be defined as important mediators of the disease. These studies lead to better treatment of the disease progression by affording the possibility of combination therapies (*e.g.*, multiple ribozymes targeted to different genes, ribozymes coupled with known small molecule inhibitors, or intermittent treatment with combinations of ribozymes and/or other chemical or biological molecules).

4.13 MAMMALIAN NERVOUS SYSTEMS

Mammalian nerve cells consist of a central portion containing the nucleus, known as the cell body, and one or more structures referred to as axons and dendrites. These dendrites are short extensions of the cell body and are involved in the reception of stimuli. In contrast, axons , are usually single elongated extensions, which are especially important in the transmission of nerve impulses from the region of the cell body to other cells. Although all many-celled animals have some kind of nervous system, the complexity of its organization varies considerably among different animal types. The cell bodies of neurons are organized in clusters called ganglia. These clusters are interconnected by the neuronal processes to form a ganglionated chain. Such chains are found in all vertebrates, in which they represent a special part of the nervous system, related especially to the regulation of the activities of the heart, the glands, and the involuntary muscles.

The peripheral nervous system is largely made up of nerves collections of cell processes, their insulating sheathes of myelin and the cells that secrete it, Schwann cells and connective tissue. At intervals, ganglia, collections of cell bodies can be found. The CNS is made up of the specialized brain, and less specialized spinal cord. The spinal cord is made up largely of tracts, their insulating sheaths of myelin and the cells that secrete it, oligodendrocytes. In places nuclei, or accumulations of gray matter (collections of cell bodies) can be found.

4.14 ANTISENSE OLIGONUCLEOTIDES

In certain embodiments, the AAV constructs of the invention will find utility in the delivery of antisense oligonucleotides and polynucleotides for inhibiting the expression of a selected mammalian mRNA in neural cells.

In the art the letters, A, G, C, T, and U respectively indicate nucleotides in which the nucleoside is Adenosine (Ade), Guanosine (Gua), Cytidine (Cyt), Thymidine (Thy), and Uridine (Ura). As used in the specification and claims, compounds that are "antisense" to a particular PNA, DNA or mRNA "sense" strand are nucleotide compounds that have a nucleoside

sequence that is complementary to the sense strand. It will be understood by those skilled in the art that the present invention broadly includes oligonucleotide compounds that are capable of binding to the selected DNA or mRNA sense strand. It will also be understood that mRNA includes not only the ribonucleotide sequences encoding a protein, but also regions including the 5'-untranslated region, the 3'-untranslated region, the 5'-cap region and the intron/exon junction regions.

The invention includes compounds which are not strictly antisense; the compounds of the invention also include those oligonucleotides that may have some bases that are not complementary to bases in the sense strand provided such compounds have sufficient binding affinity for the particular DNA or mRNA for which an inhibition of expression is desired. In addition, base modifications or the use of universal bases such as inosine in the oligonucleotides of the invention are contemplated within the scope of the subject invention.

The antisense compounds may have some or all of the phosphates in the nucleotides replaced by phosphorothioates (X=S) or methylphosphonates ($X=CH_3$) or other C_{1-4} alkylphosphonates. The antisense compounds optionally may be further differentiated from native DNA by replacing one or both of the free hydroxy groups of the antisense molecule with C_{1-4} alkoxy groups ($R=C_{1-4}$ alkoxy). As used herein, C_{1-4} alkyl means a branched or unbranched hydrocarbon having 1 to 4 carbon-atoms.

The disclosed antisense compounds also may be substituted at the 3′ and/or 5′ ends by a substituted acridine derivative. As used herein, "substituted acridine," means any acridine derivative capable of intercalating nucleotide strands such as DNA. Preferred substituted acridines are 2-methoxy-6-chloro-9-pentylaminoacridine, *N*-(6-chloro-2-methoxyacridinyl)-O-methoxydiisopropylaminophosphinyl-3-aminopropanol, and *N*-(6-chloro-2-methoxyacridinyl)-O-methoxydiisopropylaminophosphinyl-5-aminopentanol. Other suitable acridine derivatives are readily apparent to persons skilled in the art. Additionally, as used herein "P(O)(O) -substituted acridine" means a phosphate covalently linked to a substitute acridine.

As used herein, the term "nucleotides" includes nucleotides in which the phosphate moiety is replaced by phosphorothioate or alkylphosphonate and the nucleotides may be substituted by substituted acridines.

In one embodiment, the antisense compounds of the invention differ from native DNA by the modification of the phosphodiester backbone to extend the life of the antisense molecule. For example, the phosphates can be replaced by phosphorothioates. The ends of the molecule may also be optimally substituted by an acridine derivative that intercalates nucleotide strands of

DNA. Intl. Pat. Appl. Publ. No. WO 98/13526 and U. S. Patent 5,849,902 (each specifically incorporated herein by reference in its entirety) describe a method of preparing three component chimeric antisense compositions, and discuss many of the currently available methodologies for synthesis of substituted oligonucleotides having improved antisense characteristics and/or half-life.

The reaction scheme involves ^{1}H-tetrazole-catalyzed coupling of phosphoramidites to give phosphate intermediates that are subsequently reacted with sulfur in 2,6-lutidine to generate phosphate compounds. Oligonucleotide compounds are prepared by treating the phosphate compounds with thiophenoxide (1:2:2 thiophenol/triethylamine/tetrahydrofuran, room temperature, 1 hr). The reaction sequence is repeated until an oligonucleotide compound of the desired length has been prepared. The compounds are cleaved from the support by treating with ammonium hydroxide at room temperature for 1 hr and then are further deprotected by heating at about 50°C overnight to yield preferred antisense compounds.

Selection of antisense compositions specific for a given gene sequence is based upon analysis of the chosen target sequence and determination of secondary structure, T_m, binding energy, relative stability, and antisense compositions were selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA, are those that are at or near the AUG translation initiation codon, and those sequences that were substantially complementary to 5′ regions of the mRNA. These secondary structure analyses and target site selection considerations were performed using v.4 of the OLIGO primer analysis software (Rychlik, 1997) and the BLASTN 2.0.5 algorithm software (Altschul *et al.*, 1997).

4.15 EXEMPLARY DEFINITIONS

In accordance with the present invention, polynucleotides, nucleic acid segments, nucleic acid sequences, and the like, include, but are not limited to, DNAs (including and not limited to genomic or extragenomic DNAs), genes, peptide nucleic acids (PNAs) RNAs (including, but not limited to, rRNAs, mRNAs and tRNAs), nucleosides, and suitable nucleic acid segments either obtained from native sources, chemically synthesized, modified, or otherwise prepared in whole or in part by the hand of man.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and compositions similar or equivalent to those described

herein can be used in the practice or testing of the present invention, the preferred methods and compositions are described herein. For purposes of the present invention, the following terms are defined below:

A, an: In accordance with long standing patent law convention, the words "a" and "an" when used in this application, including the claims, denotes "one or more".

Expression: The combination of intracellular processes, including transcription and translation undergone by a polynucleotide such as a structural gene to synthesize the encoded peptide or polypeptide.

Promoter: a term used to generally describe the region or regions of a nucleic acid sequence that regulates transcription.

Regulatory Element: a term used to generally describe the region or regions of a nucleic acid sequence that regulates transcription.

Structural gene: A gene or sequence region that is expressed to produce an encoded peptide or polypeptide.

Transformation: A process of introducing an exogenous polynucleotide sequence (*e.g.*, a vector, a recombinant DNA or RNA molecule) into a host cell or protoplast in which that exogenous nucleic acid segment is incorporated into at least a first chromosome or is capable of autonomous replication within the transformed host cell. Transfection, electroporation, and naked nucleic acid uptake all represent examples of techniques used to transform a host cell with one or more polynucleotides.

Transformed cell: A host cell whose nucleic acid complement has been altered by the introduction of one or more exogenous polynucleotides into that cell.

Transgenic cell: Any cell derived or regenerated from a transformed cell or derived from a transgenic cell, or from the progeny or offspring of any generation of such a transformed host cell.

Vector: A nucleic acid molecule (typically comprised of DNA) capable of replication in a host cell and/or to which another nucleic acid segment can be operatively linked so as to bring about replication of the attached segment. A plasmid, cosmid, or a virus is an exemplary vector.

The terms "substantially corresponds to", "substantially homologous", or "substantial identity" as used herein denotes a characteristic of a nucleic acid or an amino acid sequence, wherein a selected nucleic acid or amino acid sequence has at least about 70 or about 75 percent sequence identity as compared to a selected reference nucleic acid or amino acid sequence. More typically, the selected sequence and the reference sequence will have at least

about 76, 77, 78, 79, 80, 81, 82, 83, 84 or even 85 percent sequence identity, and more preferably at least about 86, 87, 88, 89, 90, 91, 92, 93, 94, or 95 percent sequence identity. More preferably still, highly homologous sequences often share greater than at least about 96, 97, 98, or 99 percent sequence identity between the selected sequence and the reference sequence to which it was compared. The percentage of sequence identity may be calculated over the entire length of the sequences to be compared, or may be calculated by excluding small deletions or additions which total less than about 25 percent or so of the chosen reference sequence. The reference sequence may be a subset of a larger sequence, such as a portion of a gene or flanking sequence, or a repetitive portion of a chromosome. However, in the case of sequence homology of two or more polynucleotide sequences, the reference sequence will typically comprise at least about 18-25 nucleotides, more typically at least about 26 to 35 nucleotides, and even more typically at least about 40, 50, 60, 70, 80, 90, or even 100 or so nucleotides. Desirably, which highly homologous fragments are desired, the extent of percent identity between the two sequences will be at least about 80%, preferably at least about 85%, and more preferably about 90% or 95% or higher, as readily determined by one or more of the sequence comparison algorithms well-known to those of skill in the art, such as e.g., the FASTA program analysis described by Pearson and Lipman (1988).

The term "naturally occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by the hand of man in a laboratory is naturally-occurring. As used herein, laboratory strains of rodents that may have been selectively bred according to classical genetics are considered naturally occurring animals.

As used herein, a "heterologous" is defined in relation to a predetermined referenced gene sequence. For example, with respect to a structural gene sequence, a heterologous promoter is defined as a promoter which does not naturally occur adjacent to the referenced structural gene, but which is positioned by laboratory manipulation. Likewise, a heterologous gene or nucleic acid segment is defined as a gene or segment that does not naturally occur adjacent to the referenced promoter and/or enhancer elements.

"Transcriptional regulatory element" refers to a polynucleotide sequence that activates transcription alone or in combination with one or more other nucleic acid sequences. A transcriptional regulatory element can, for example, comprise one or more promoters, one or more response elements, one or more negative regulatory elements, and/or one or more enhancers.

As used herein, a "transcription factor recognition site" and a "transcription factor binding site" refer to a polynucleotide sequence(s) or sequence motif(s) which are identified as being sites for the sequence-specific interaction of one or more transcription factors, frequently taking the form of direct protein-DNA binding. Typically, transcription factor binding sites can be identified by DNA footprinting, gel mobility shift assays, and the like, and/or can be predicted on the basis of known consensus sequence motifs, or by other methods known to those of skill in the art.

As used herein, the term "operably linked" refers to a linkage of two or more polynucleotides or two or more nucleic acid sequences in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. However, since enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not contiguous.

"Transcriptional unit" refers to a polynucleotide sequence that comprises at least a first structural gene operably linked to at least a first *cis*-acting promoter sequence and optionally linked operably to one or more other *cis*-acting nucleic acid sequences necessary for efficient transcription of the structural gene sequences, and at least a first distal regulatory element as may be required for the appropriate tissue-specific and developmental transcription of the structural gene sequence operably positioned under the control of the promoter and/or enhancer elements, as well as any additional *cis* sequences that are necessary for efficient transcription and translation (*e.g.*, polyadenylation site(s), mRNA stability controlling sequence(s), *etc.*

The term "substantially complementary," when used to define either amino acid or nucleic acid sequences, means that a particular subject sequence, for example, an oligonucleotide sequence, is substantially complementary to all or a portion of the selected sequence, and thus will specifically bind to a portion of an mRNA encoding the selected sequence. As such, typically the sequences will be highly complementary to the mRNA "target" sequence, and will have no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 base mismatches throughout the complementary portion of the sequence. In many instances, it may be desirable for the sequences to be exact matches, *i.e.* be completely complementary to the sequence to

which the oligonucleotide specifically binds, and therefore have zero mismatches along the complementary stretch. As such, highly complementary sequences will typically bind quite specifically to the target sequence region of the mRNA and will therefore be highly efficient in reducing, and/or even inhibiting the translation of the target mRNA sequence into polypeptide product.

Substantially complementary oligonucleotide sequences will be greater than about 80 percent complementary (or '% exact-match') to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and will, more preferably be greater than about 85 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds. In certain aspects, as described above, it will be desirable to have even more substantially complementary oligonucleotide sequences for use in the practice of the invention, and in such instances, the oligonucleotide sequences will be greater than about 90 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and may in certain embodiments be greater than about 95 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and even up to and including 96%, 97%, 98%, 99%, and even 100% exact match complementary to all or a portion of the target mRNA to which the designed oligonucleotide specifically binds.

Percent similarity or percent complementary of any of the disclosed sequences may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0, available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (1970). Briefly, the GAP program defines similarity as the number of aligned symbols (*i.e.*, nucleotides or amino acids) that are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess (1986), (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

5. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in

the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5.1 EXAMPLE 1 – AAV5 SEROTYPE VECTORS ARE MORE EFFICIENT THAN AAV2 IN TRANSDUCTION OF NEURAL CELLS

It has been reported that the rAAV5 serotype leads to greater expression levels and a wider transduction area than the "standard" rAAV2 serotype. In support of this possibility, the inventors produced rAAV5-CBA-GFP and compared the striatal transduction efficiency of this vector directly to rAAV2-CBA-GFP. As demonstrated in FIG. 1A-FIG. 1I, rAAV serotype 5 vectors supported much higher striatal transduction efficiency than rAAV2-based vectors. Presumably the more efficient transduction of rAAV5 serotype vectors is due to the fact that they use sialic acid as one of their receptors, whereas rAAV2 vectors bind heparin sulfate proteoglycan. The rAAV5 vector shown in FIG. 1A-FIG. 1I contains the same chicken beta-actin-green fluorescent protein (CBA-GFP) vector construct, containing AAV2 TRs that was packaged in the rAAV2 serotype vector. This was accomplished by using a complementing plasmid that expressed AAV2 rep and AAV5 cap and it allowed direct comparison of the capsid serotype effect on striatal transduction efficiency.

5.2 EXAMPLE 2 – AAV1 SEROTYPE VECTORS ARE MORE EFFICIENT THAN AAV2 IN TRANSDUCTION OF NEURAL CELLS

In support of the finding that rAAV1 serotype vectors lead to greater expression levels and a wider transduction area than the "standard" rAAV2 serotype, the inventors have also produced rAAV1-CBA-GFP and compared the striatal transduction efficiency of this vector directly to rAAV2-CBA-GFP. As demonstrated in FIG. 2A and FIG. 2B, rAAV1 also supported much higher striatal transduction efficiency than rAAV2-based vectors, demonstrating its usefulness in transfection of select mammalian neural cells. FIG. 3A, FIG. 3B, FIG. 3C, and FIG. 3D show the results of injection of the rAAV1-GFP and rAAV2-GFP constructs in the striatum and the hippocampus. FIG. 4A, FIG. 4B, FIG. 4C, and FIG. 4D show the results of injection of the rAAV1-GFP and rAAV2-GFP constructs in the globus pallidus and the substantia nigra.

6. REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference in whole or in part:

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All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

WHAT IS CLAIMED IS:

1. A recombinant adeno-associated viral serotype 1 (rAAV1) or an adeno-associated viral serotype 5 (rAAV5) vector comprising: at least a first neurotherapeutic expression unit that comprises at least a first nucleic acid segment encoding a neurotherapeutic agent operably linked to a promoter that expresses said segment in a mammalian neural cell, brain cell, central or peripheral nervous system cell that comprises said vector.

2. The recombinant adeno-associated viral vector of claim 1, wherein said neurotherapeutic agent comprises at least a first neurotherapeutic protein, polypeptide or peptide.

3. The recombinant adeno-associated viral vector of claim 2, wherein said neurotherapeutic protein, polypeptide, or peptide is selected from the group consisting of a neurotrophic factor, a cytokine, a cytotoxin, a tumor suppressor, an anti-apoptotic factor, a growth factor, a cytokine receptor, a growth factor receptor, an interferon, a semaphorin, a plexin, a neuropilin, a netrin, a serotonin transport protein, a glutamic acid decarboxylase, a protein kinase, a protein kinase inhibitor, a glycoprotein, a hormone, a proteolytic protein, a neurogenic factor, a growth factor, a neurotrophin, an apoptosis inhibitor, an adrenergic agonist, an erythropoietic agent, an N-methyl-D-aspartate antagonist, a nerve growth factor, a neuroactive peptide receptor, and a neurotrophin receptor.

4. The recombinant adeno-associated viral vector of claim 2, wherein said promoter is a heterologous, tissue-specific constitutive or inducible promoter.

5. The recombinant adeno-associated viral vector of claim 2, wherein said promoter is selected from the group consisting of a brain-specific, a neural specific, a central nervous system specific, and a peripheral nervous system cell-specific promoter.

6. The recombinant adeno-associated viral vector of claim 5, wherein said neural-specific promoter is selected from the group consisting of a BDNF promoter, an NGF promoter, a growth factor promoter, an axon-specific promoter, a dendrite-specific promoter, a brain-specific promoter, and a hippocampal-specific promoter.

7. The recombinant adeno-associated viral vector of claim 2, wherein said promoter is selected from the group consisting of a CMV promoter, a β-actin promoter, a cytokine promoter, a growth factor promoter, a neuron-specific promoter, an enolase promoter, a neurotrophin promoter, a hybrid CMV promoter, a hybrid β-actin promoter, an EF1 promoter, a U1a promoter, a U1b promoter, a Tet-inducible promoter and a VP16-LexA promoter.

8. The recombinant adeno-associated viral vector of claim 7, wherein said promoter is a mammalian β-actin promoter.

9. The recombinant adeno-associated viral vector of claim 8, wherein said promoter is a chicken β-actin promoter.

10. The recombinant adeno-associated viral vector of claim 1, wherein said neurotherapeutic expression unit further comprises an enhancer sequence operably linked to said nucleic acid segment.

11. The recombinant adeno-associated viral vector of claim 10, wherein said enhancer sequence comprises a CMV enhancer, a synthetic enhancer, a neural-specific enhancer, a brain specific-specific enhancer, or a neuron-specific enhancer.

12. The recombinant adeno-associated viral vector of claim 1, wherein said nucleic acid segment further comprises a post-transcriptional regulatory sequence.

13. The recombinant adeno-associated viral vector of claim 12, wherein said regulatory sequence comprises a woodchuck hepatitis virus post-transcription regulatory element.

14. The recombinant adeno-associated viral vector of claim 1, wherein said at least a first neurotherapeutic expression unit comprises at least a first nucleic acid segment encoding a ribozyme, an antisense oligonucleotide, or a therapeutic protein, polypeptide or peptide operably linked to a neural cell-specific promoter that expresses said segment in a mammalian neural, brain, central or peripheral nervous system cell containing said vector.

15. The recombinant adeno-associated viral of claim 1, wherein said neurotherapeutic agent is a peptide or polypeptide of human, primate, murine, porcine, bovine, ovine, feline, canine, equine, epine, caprine, or lupine origin.

16. The recombinant adeno-associated viral of claim 1, wherein said mammalian cell is a human, primate, murine, feline, canine, porcine, ovine, bovine, equine, epine, caprine, or lupine cell.

17. The recombinant adeno-associated viral vector of claim 1, wherein said vector further comprises at least a first enhancer.

18. The recombinant adeno-associated viral vector of claim 17, wherein said vector further comprises a CMV enhancer, a synthetic enhancer, a brain-specific enhancer, a

neural cell-specific enhancer, or a peripheral or central nervous system cell-specific enhancer.

19. The recombinant adeno-associated viral vector of claim 1, wherein said vector further comprises at least a first intron sequence.

20. The recombinant adeno-associated viral vector of claim 1, further comprising at least a second nucleic acid segment that encodes at least a second peptide that specifically targets a virion or viral particle expressing said vector to the cell surface of a mammalian neural, brain, central or peripheral nervous system cell.

21. The recombinant adeno-associated viral vector of claim 20, wherein said second nucleic acid segment encodes a second peptide that specifically targets said virion or said viral particle expressing said vector to a cell surface receptor polypeptide of said mammalian neural, brain, central or peripheral nervous system cell.

22. The recombinant adeno-associated viral vector of claim 1, comprised within an adeno-associated viral particle.

23. The recombinant adeno-associated viral vector of claim 1, comprised within a pharmaceutical vehicle.

24. The recombinant adeno-associated viral vector of claim 23, formulated for administration to a human.

25. A recombinant adeno-associated virus virion comprising the recombinant adeno-associated viral vector of claim 1.

26. A plurality of adeno-associated viral particles comprising the vector of claim 1.

27. A mammalian cell comprising the vector of claim 1, or the recombinant adeno-associated virus virion of claim 25.

28. The mammalian cell of claim 27, wherein said cell is a human neural cell, brain cell, or central or peripheral nervous system cell cell.

29. The mammalian cell of claim 27, wherein said cell is a human globus pallidus, substantia nigra, hippocampal, or striatal cell of the brain tissue of said mammal.

30. A composition comprising the vector of claim 1, the recombinant adeno-associated virus virion of claim 25, the plurality of adeno-associated viral particles of claim 26; or the mammalian cell of claim 27.

31. The composition of claim 30, further comprising a pharmaceutical excipient, buffer, or diluent.

32. The composition of claim 31, formulated for administration to a human.

33. The composition of claim 30, further comprising a liposome, a lipid, microsphere, nanosphere, nanocapsule, microcapsule, or a lipid complex.

34. The composition of claim 30, for use in therapy.

35. The composition of claim 34, for use in neuromuscular, neurological, neurocognitive, neuroskeletal, or neurosensory therapy.

36. Use of a composition according to claim 30, in the manufacture of a medicament for treating cancer, stroke, ischemia, neuromuscular disorders, neurological diseases, neuroskeletal impairment, or neurosensory disability or dysfunction.

37. Use according to claim 36, in the manufacture of a medicament for treating human brain or neural dysfunction.

38. A kit comprising:

(a) the adeno-associated viral vector of claim 1, the virion of claim 250, the viral particles of claim 26, the cell of claim 27, or the composition of claim 30; and

(b) instructions for using said kit.

39. A method for producing a biologically-effective amount of an expressed neurotherapeutic agent in neural cells of a mammal in need thereof, said method comprising providing to said mammal the composition of claim 30, in an amount and for a time sufficient to produce said neurotherapeutic agent in said neural cells of said mammal.

40. The method of claim 39, wherein said expressed neurotherapeutic agent is a polypeptide, peptide, or protein.

41. The method of claim 40, wherein said expressed neurotherapeutic agent is a protein, polypeptide, or peptide selected from the group consisting of a neurotrophic factor, a cytokine, a cytotoxin, a tumor suppressor, an anti-apoptotic factor, a growth factor, a cytokine receptor, a growth factor receptor, an interferon, a semaphorin, a plexin, a neuropilin, a netrin, a serotonin transport protein, a glutamic acid decarboxylase, a protein kinase, a protein kinase inhibitor, a glycoprotein, a hormone, a proteolytic protein, a neurogenic factor, a growth factor, a neurotrophin, an apoptosis inhibitor, an adrenergic agonist, an erythropoietic agent, an N-methyl-D-aspartate antagonist, a nerve growth factor, a neuroactive peptide receptor, and a neurotrophin receptor.

42. The method of claim 40, wherein said mammal is a human.

43. The method of claim 42, wherein said mammal is a human having, at risk for developing, or suspected of having a deficiency or mutation in one or more proteins, polypeptides, or peptides normally present in at least a first neural cell of said mammal.

44. The method of claim 40, wherein said composition is provided to said mammal in an amount and for a time sufficient to treat, prevent, or ameliorate the symptoms of at least a first neurological disorder or neural dysfunction in said mammal.

45. The method of claim 44, wherein said composition is provided to said mammal intramuscularly, intravenously, subcutaneously, intrathecally, intraperitoneally, intracerebroventricularly, or by stereotactic injection into the brain, spine, CNS, PNS, or a nerve cell or neural tissue.

46. A method for reducing the level of a selected polypeptide in a mammalian neural cell, said method comprising providing to said neural cell, an amount of a composition that

comprises a recombinant adeno-associated viral serotype 1 (rAAV1) or an adeno-associated viral serotype 5 (rAAV5) vector comprising: a nucleic acid segment encoding an antisense molecule or catalytic ribozyme that specifically binds to a nucleic acid segment encoding said selected polypeptide for a time effective to reduce the level of said polypeptide in said mammalian neural cell.

47. A method for reducing the transcription of a selected mRNA in a mammalian neural cell, said method comprising providing to said neural cell, an amount of a composition that comprises a recombinant adeno-associated viral serotype 1 (rAAV1) or an adeno-associated viral serotype 5 (rAAV5) vector comprising: a nucleic acid segment encoding an antisense molecule or catalytic ribozyme that specifically binds to said selected mRNA, for a time effective to reduce the transcription of said mRNA in said mammalian neural cell.

48. A method for preventing, treating or ameliorating the symptoms of a neural disease, dysfunction, or deficiency in a mammal, said method comprising providing to neural cells of said mammal, the vector of claim 1, the virion of claim 25, or the viral particles of claim 26, in an amount and for a time sufficient to treat or ameliorate the symptoms of said disease, dysfunction, or deficiency in said mammal.

49. The method of claim 48, wherein said mammal is a human.

50. The method of claim 48, wherein said mammal has, is diagnosed with, or is at risk for developing a neural impairment, a neurosensory disorder, a neuromuscular disease, cancer, a tumor, or metastatic growth of the brain or nervous system, memory loss, age-related memory loss, neurocognitive disease, a socioaffective disorder, autism, ALS, cerebral palsy, ischemia, cerebrovascular injury, Alzheimer's disease, Parkinson's disease, Huntington's disease, Tay-Sach's disease, Niemann-Pick's disease, Guillain-Barre syndrome, seizures, coma, dementia, schizophrenia, brain injury, a loss of comprehension, a learning disability, or sensory motor impairment.

51. The method of claim 48, wherein said vector, said virion, or said plurality of viral particles is administered to said mammal intracerebrally, intracerebroventricularly, intramuscularly, intravenously, subcutaneously, intrathecally, intraperitoneally, or by direct injection into an organ, tissue, or cell type.

52. The method of claim 51, wherein said vector, said virion, or said plurality of viral particles is administered to the globus pallidus, the substantia nigra, the hippocampus, or the striatum of the brain tissue of said mammal.

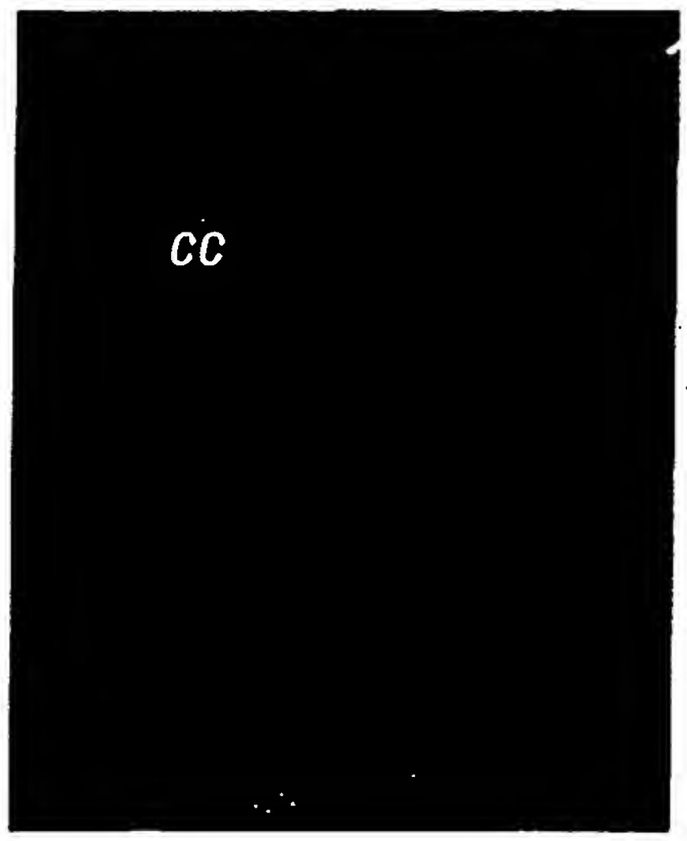

FIG. 1A

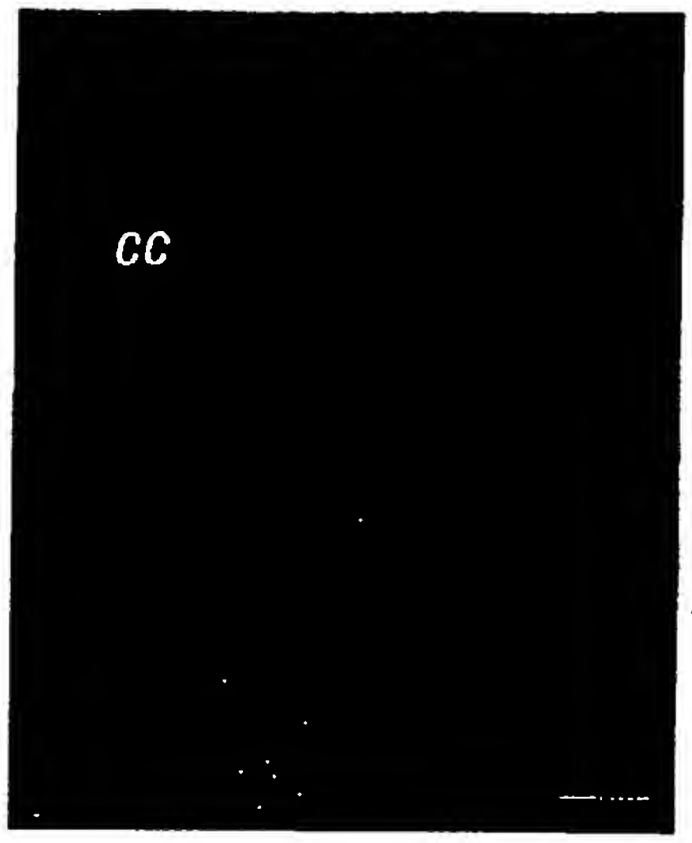

FIG. 1B

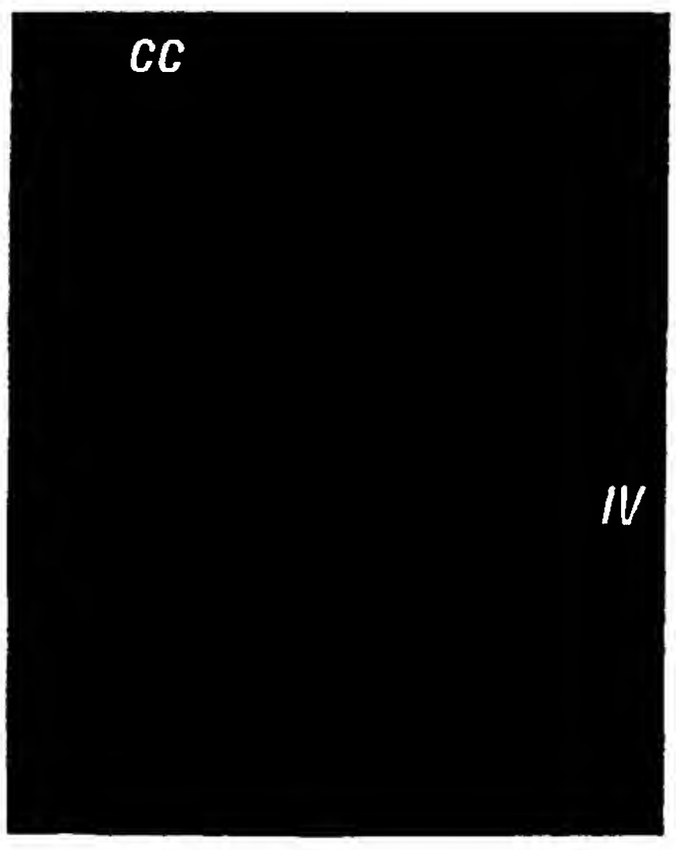

FIG. 1C

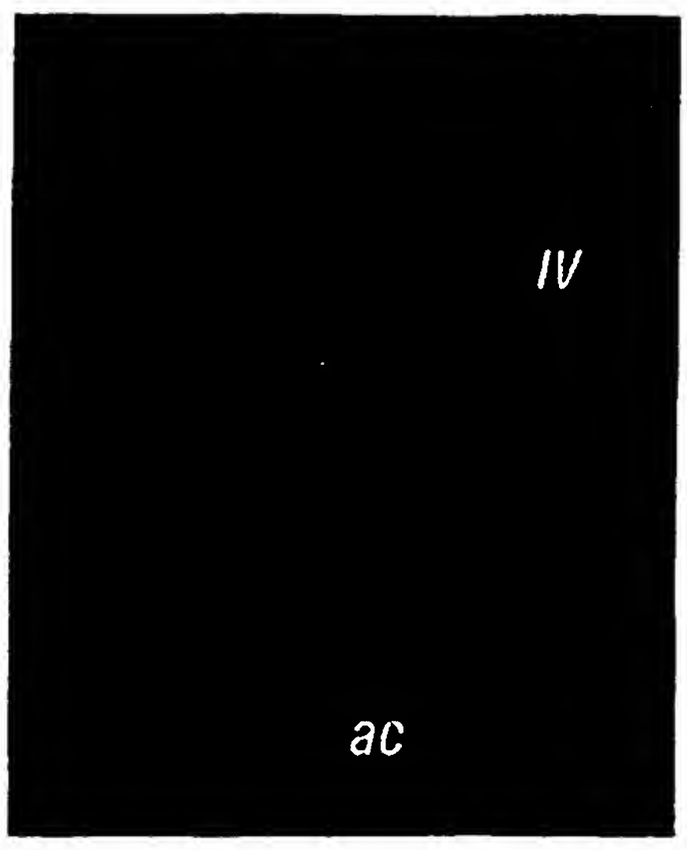

FIG. 1D

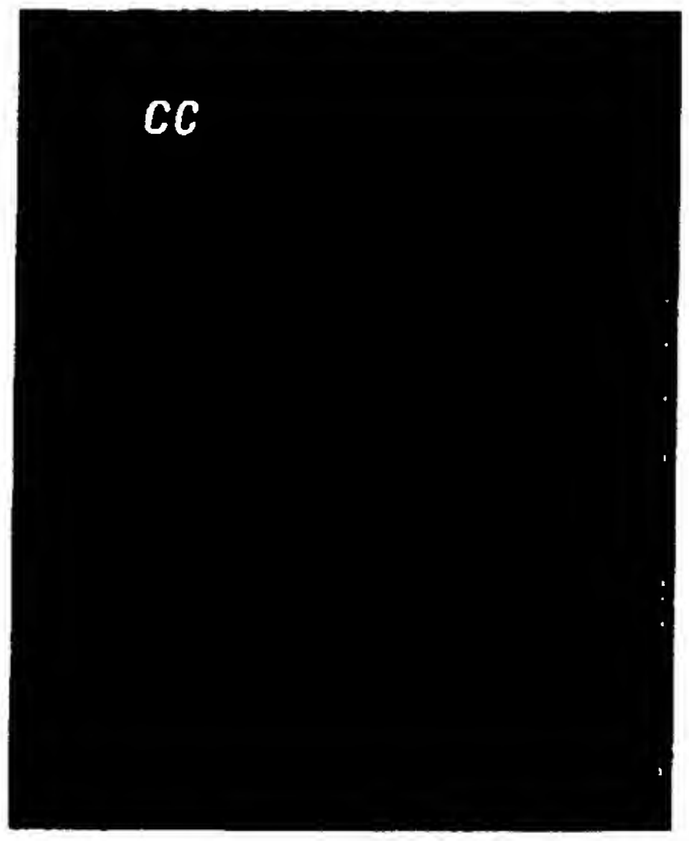

FIG. 1E

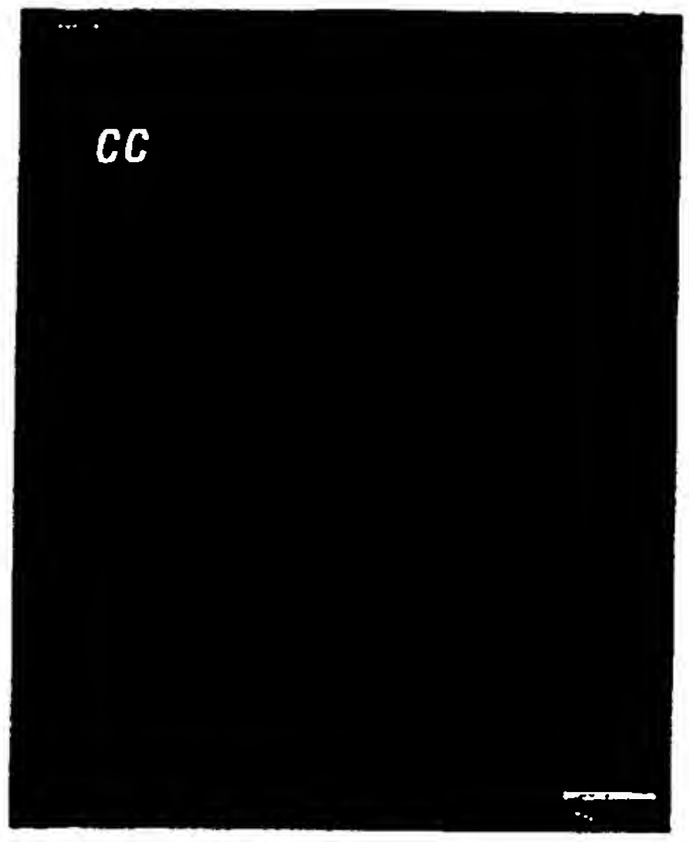

FIG. 1F

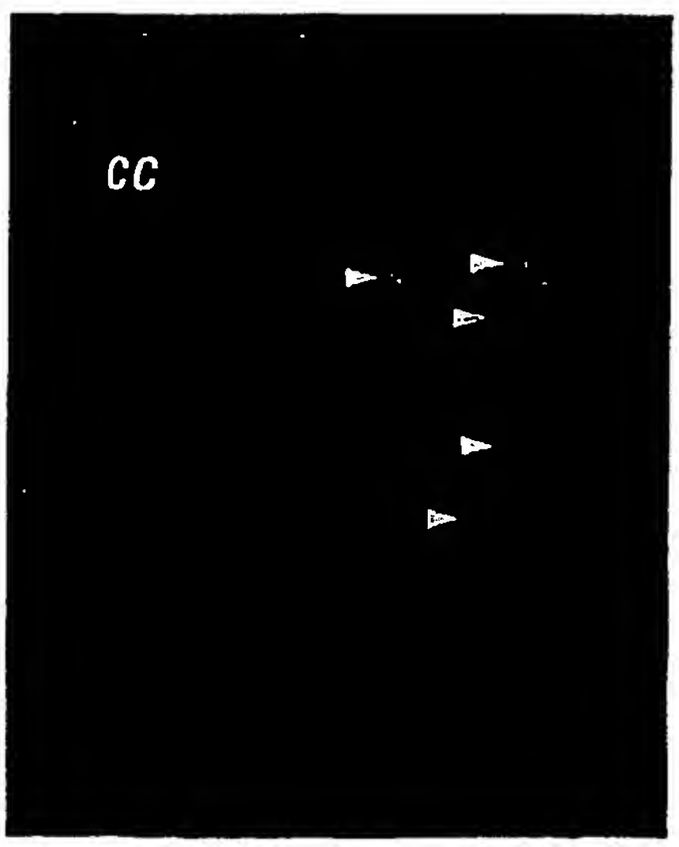

FIG. 1G

FIG. 1H

FIG. 1I

FIG. 2A

FIG. 2B

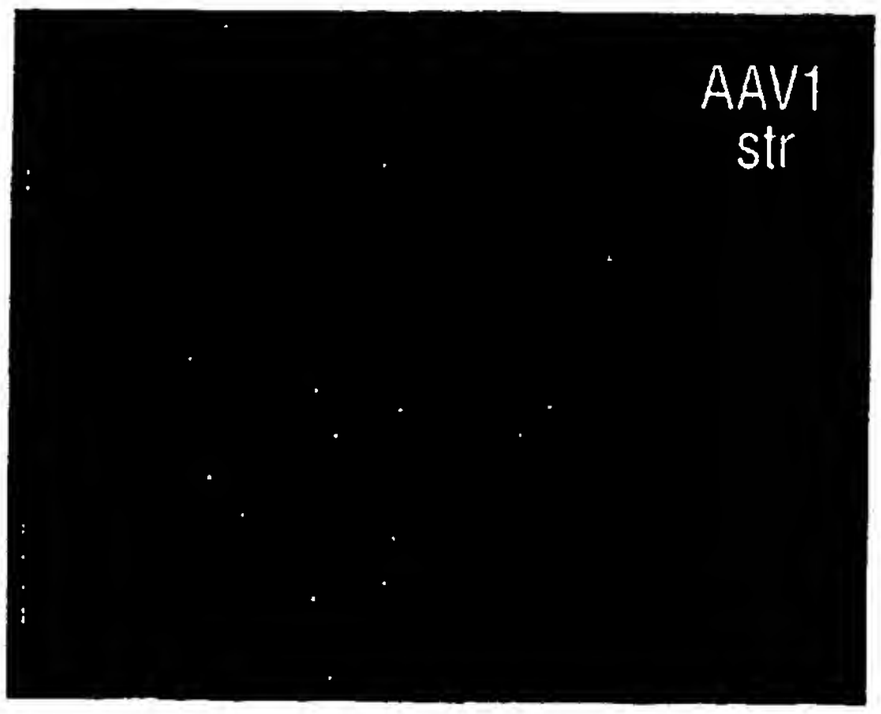

FIG. 3A

FIG. 3B

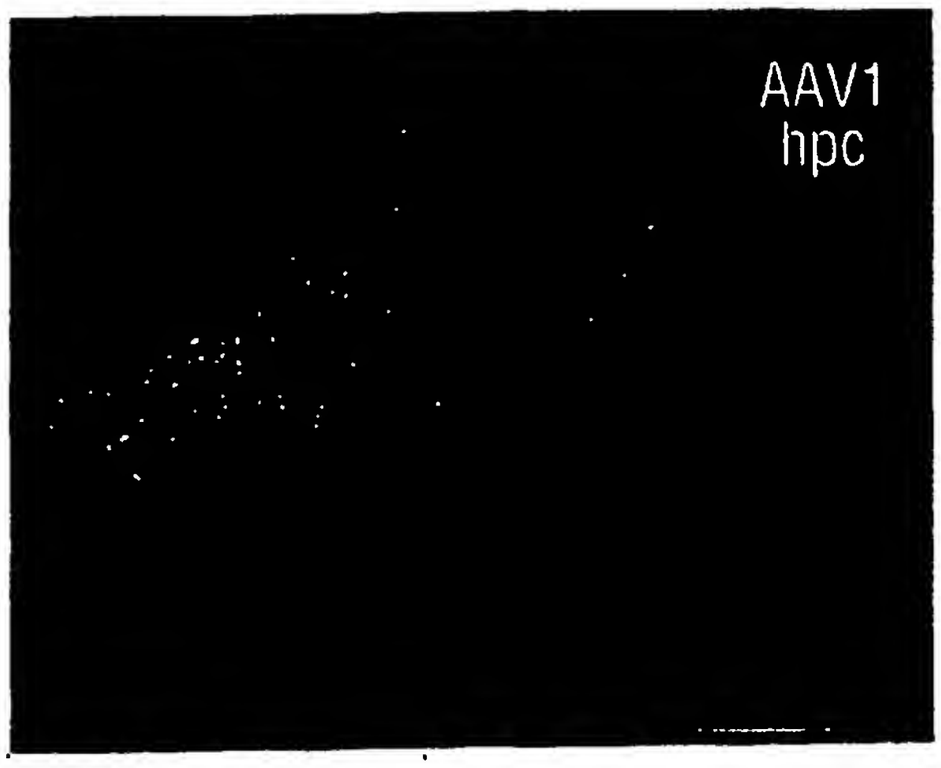

FIG. 3C

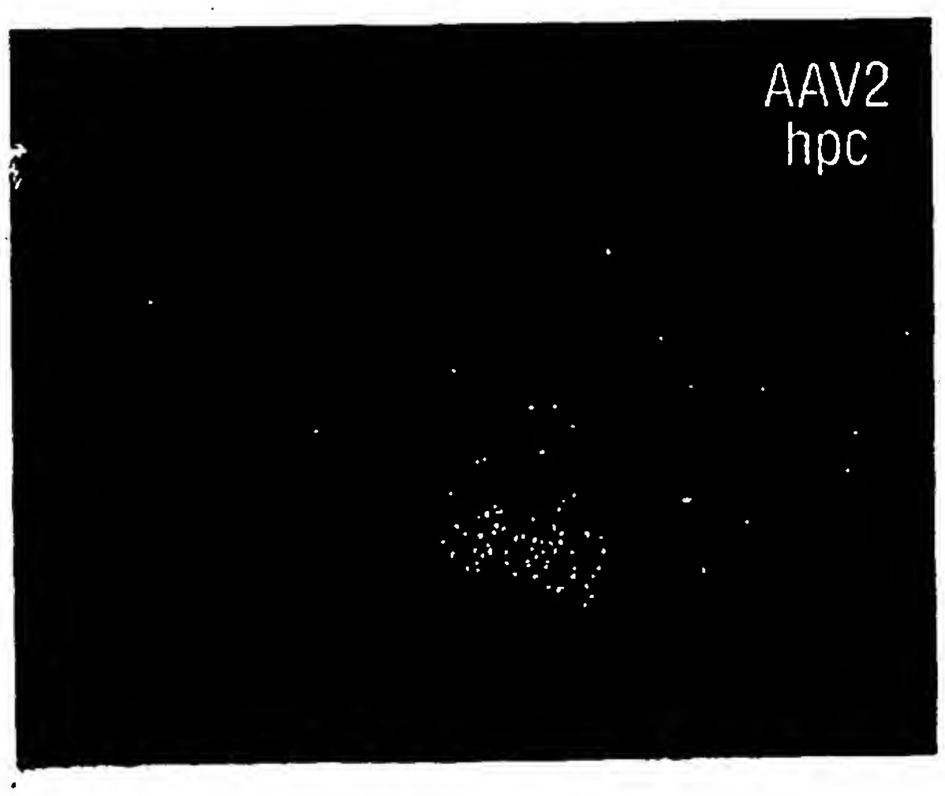

FIG. 3D

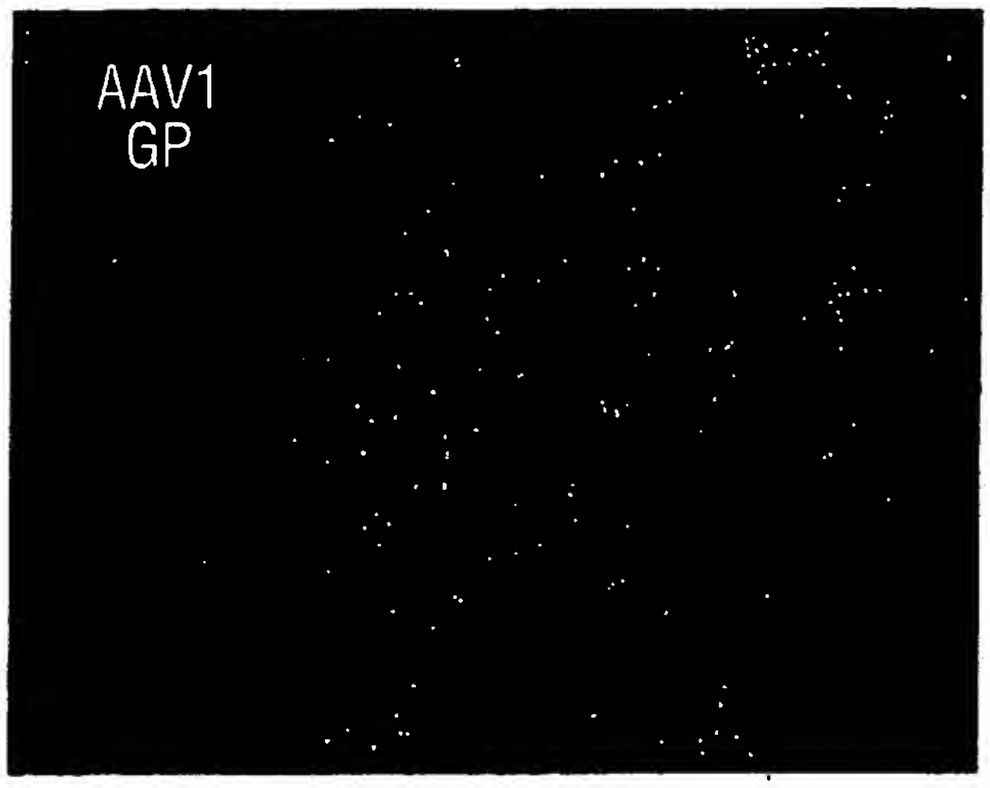

FIG. 4A

FIG. 4B

FIG. 4C

FIG. 4D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/13592

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12N 15/63, 15/85 ,15/87; A01N 43/04; A61K 31/70
US CL : 435/455;514/44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/455;514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,952,221 A (KURTZMAN et al) 14 September 1999 (14.09.1999) see whole document, especially columns 4-13.	1-7, 10-12, 14-18, 22-45, 48-52 --- 20,21,46,47
A,E	PASSINI et al. Intraventricular brain injection of adeno-associated virus type 1 (AAV1) in neonatal mice results in complementary patterns of neuronal transduction to AAV2 and total long-term correction of storage lesions in the brains of beta-glucuronidase-deficient mice. Journal of Virology. June 2003, Vol. 77, No. 12, pages 7034-7040.	1-7, 10-12, 14-18, 21-45, 48-52
Y,P	US 2002/0131961 A1 (WILSON et al) 19 September 2002 (19.09.2002) see whole document, especially pages 3 and 8.	1, 2, 7- 11, 17, 18
Y	XIAO et al. Gene therapy vectors based on adeno-associated virus type 1. Journal of Virology. May 1999, Vol. 73, No. 5, pages 3994-4003, see entire document.	1-52
Y	MCGEE SANFTNER et al. Glial cell line derived neurotrophic factor delays photoreceptor degeneration in a transgenic rat model of retinitis pigmentosa. Molecular Therapy. December 2001, Vol. 4, No. 6, pages 622-629, see entire document.	1,7-9

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 August 2003 (07.08.2003)	02 SEP 2003
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230	Authorized officer Brian Whiteman Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

PCT/US03/13592

INTERNATIONAL SEARCH REPORT

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PATERNA et al. Influence of promoter and WHV post-transcriptional regulatory element on AAV-mediated transgene expression in the rat brain. Gene Therapy. 2000 Vol. 7 pages 1304-1311, see entire document.	1,12,13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/13592

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

PCT/US03/13592

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions, which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-38, drawn to a recombinant adeno-associated viral serotype 1 (rAAV1) or an adeno-associated viral serotype 5 (rAAV5) vector comprising: a neurotherapeutic expression unit that comprises a nucleic acid sequence encoding a neurotherapeutic protein operably linked to a promoter.

Group II, claim(s) 39 and 46-47, drawn to a method of reducing the transcription of a selected mRNA in a mammalian neural cell comprising a administering a recombinant adeno-associated viral serotype 1 (rAAV1) or an adeno-associated viral serotype 5 (rAAV5) vector comprising: a neurotherapeutic expression unit that comprises an antisense molecule or catalytic ribozyme.

Group III, claim(s) 39-45 and 48-52, drawn to a method of preventing, treating, or ameliorating the symptoms of a neural disease in a mammal, said method comprising administering a recombinant adeno-associated viral serotype 1 (rAAV1) or an adeno-associated viral serotype 5 (rAAV5) vector comprising: a neurotherapeutic expression unit that comprises a nucleic acid sequence encoding a neurotherapeutic protein operably linked to a promoter.

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
The technical feature linking groups I-III appears to be that they all relate to a rAAV1 or rAAV5 vector comprising a nucleic acid sequence encoding a neurotherapeutic protein.
However, US 5,952,221 teaches a recombinant AAV-1 or AAV-5 virions containing a drug-susceptibility gene and a second gene capable of providing an ancillary effect in solid tumor cells.
Therefore, the technical feature linking the inventions of groups I-III does not constitute a special technical feature as defined by PCT Rule 13.2, as it does not define a contribution over the prior art.

The special technical feature of Group I is considered to be a recombinant rAAV-1 or rAAV-5 vector comprising a nucleotide sequence encoding a neurotherapeutic protein.
The special technical feature of Group II is considered to be a method of reducing the transcription of a selected mRNA in mammalian neural cell comprising administering a recombinant rAAV-1 or rAAV-5 vector comprising an antisense molecule or catalytic ribozyme.
The special technical feature of Group III is considered to be a method of treating the symptoms of a neural disease in a mammal, said method comprising providing to neural cells of said mammal, a recombinant rAAV-1 or rAAV-5 vector comprising a nucleotide sequence encoding a neurotherapeutic protein operably linked to a promoter.

Accordingly, Groups I-III are not so linked by the same or a corresponding special technical feature as to form a single general inventive concept.

INTERNATIONAL SEARCH REPORT

PCT/US03/13592

Continuation of B. FIELDS SEARCHED Item 3:
WEST2.1, STN
search terms: AA1, AAV5, adeno associated virus, adeno associated viral, brain, nervous system, neural, neuron, gene therapy, beta-actin promoter, woodchuck hepatitis virus post-transcriptional regulatory element